"DESIGN, SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF SOME NOVEL ANTI TUBERCULAR AGENTS TARGETING GLUTAMINE SYNTHETASE 1 "

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IN

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CERTIFICATE

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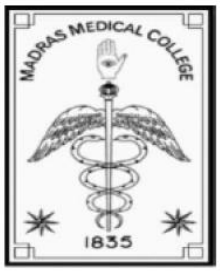

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LIST OF ABBERIVATIONS

TB	Tubercle Bacillus
HIV	Human Immuno Deficiency Disease
AIDS	Acquired Immuno Deficiency Syndrome
BCG	Bacilli Chalmette Guerin
DOTS	Directly Observed Treatment Short-Course
MDR-TB	Multi Drug Resistant-TB
XDR-TB	Extensively Drug Resistant-TB
LTBI	Latent Tuberculosis Infection
GS	Glutamine Synthetase
CADD	Computer Aided Drug Design
LC-MS	Liquid Chromatography Mass Spectroscopy
QSAR	Quantitative Structure Activity Relationship
ADME	Absorption, Distribution, Metabolism, Excretion
PSA	Polar Surface Area
OSIRIS	Optical,Spectroscopic and Infrared Remote Imaging System
OPLS	Optimized Potential For Liquid Simulation
TPSA	Total Polar Surface Area
SBDD	Structure Based Drug Design
LBDD	Ligand Based Drug Design
Log P	Partition Coefficient
WHO	World Health Organization

MIC	Minimum Inhibitory Concentration
PDB	Protein Data Bank
TLC	Thin Layer Chromatoghraphy
IR	Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
GC-MS	Gas Chromatography and Mass Spectroscopy
LC-MS	Liquid Chromatoghaphy and mass Spectroscopy
REMA	Resazurin Micro Plate Assay
MABA	MicroPlate Alamar Blue Assay
NRA	Nitrate Reductase Assay

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INTRODUCTION

The latest world health organization (WHO) reports show that there were 9.0 million new tuberculosis (TB) cases and 1.5 million tuberculosis (TB) deaths, leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). Co-infection with the HIV fuels the global TB crisis, and successful TB treatment is further complicated and hampered by the existence of multidrug- resistant (MDR) TB and extensively drug resistant (XDR) TB [1]

Tuberculosis, MTB, or TB (short for tubercle bacillus), in the past also called phthisis, phthisis pulmonalis, or consumption, is a widespread, infectious disease caused by various strains of mycobacteria, usually Mycobacterium tuberculosis. Tuberculosis generally affects the lungs, but can also affect other parts of the body. [2]

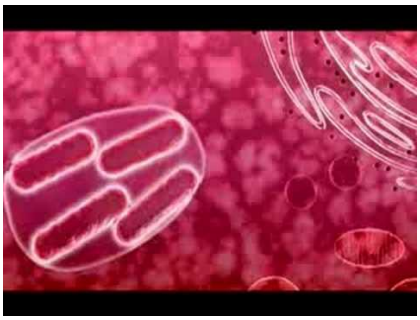

Fig 1: Mycobacterium tuberculosis

One-third of the world's population is thought to have been infected with M. tuberculosis, and new infections occur in about 1% of the population each year. In 2007, an estimated 13.7 million chronic cases were active globally, while in 2013, an estimated 9 million new cases occurred. In 2013 there were between 1.3 and 1.5 million associated deaths [2], most of which occurred in developing countries. The total number of tuberculosis cases has been decreasing since 2006, and new cases have decreased since 2002. Many people in the developing world contract tuberculosis because of a poor immune system, largely due to high rates of HIV infection and the corresponding development of AIDS. [1]

HISTORY

Tuberculosis has been present in humans since antiquity [3]. The earliest detection of M. tuberculosis involves evidence of the disease in the remains of bison in Wyoming dated to around 17,000 years ago [4]. However, whether tuberculosis originated in bovines, then was transferred to humans, or whether it diverged from a common ancestor, is currently unclear [5]. A comparison of the genes of M. tuberculosis complex (MTBC) in humans to MTBC in animals suggests humans did not acquire MTBC from animals during animal domestication, as was previously believed. Both strains of the tuberculosis bacteria share a common ancestor, which could have infected humans as early as the Neolithic Revolution.[5]

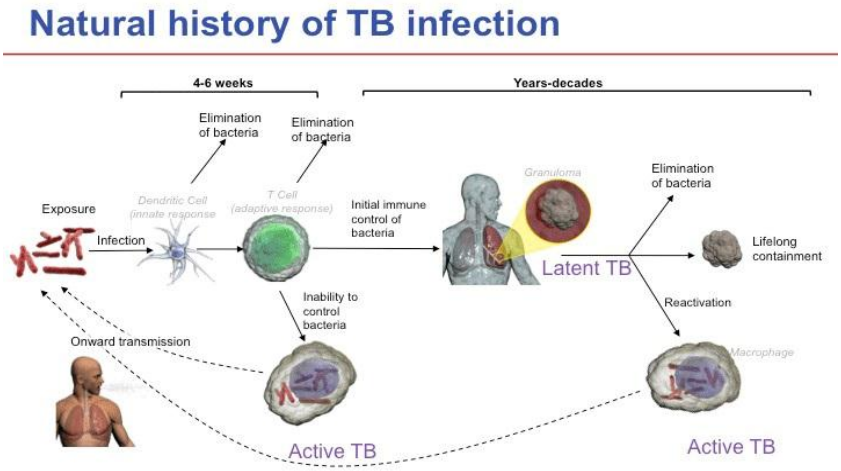

Fig 2: Natural History of TB infection

Phthisi is a Greek word for consumption, an old term for pulmonary tuberculosis; around 460 BC, Hippocrates described phthisis as a disease of dry seasons. [6]

Although the pulmonary form associated with tubercles was established as a pathology by Dr. Richard Morton in 1689 [5] [6], due to the variety of its symptoms, TB was not identified as a single disease until the 1820s. It was not named "tuberculosis"

until 1839, by J. L. Schönlein.[7][8]. The bacillus causing tuberculosis, M. tuberculosis, was identified and described on 24 March 1882 by Robert Koch.[12]

Albert Calmette and Camille Guérin achieved the first genuine success in immunization against tuberculosis in 1906, using attenuated bovine-strain tuberculosis. It was called bacille Calmette–Guérin (BCG). The BCG vaccine was first used on humans in 1921 in France[11], but received widespread acceptance in the US, Great Britain, and Germany only after World War II.[12]

TYPES OF TUBERCULOSIS

Tuberculosis is a contagious disease; it affects almost all the important organs of the body. Clinically, tuberculosis is broadly categorized into three major categories

1. Primary tuberculosis.
2. Secondary tuberculosis.
3. Disseminated tuberculosis.

Primary tuberculosis:

When tuberculosis affects a person who had never been exposed to the bacterium earlier, the condition is called primary tuberculosis. In this form of tuberculosis the source of bacterium is external.[9]

Secondary tuberculosis:

It is also known as post primary tuberculosis. This type of tuberculosis occurs in a person who previously had TB. In primary TB, the bacterium goes into an inactive face while in secondary TB the bacterium regain its active mode and causes the symptom[9]. Secondary tuberculosis more infectious than primary TB. Secondary TB increases the chance of the infections spread to other organs such as kidneys, heart, and brain.

Disseminated tuberculosis:

Disseminated means that the tuberculosis has infected the entire body system. It is a very rare type of disease. It primarily affects the bones of spines, hips, joints and knees and even the central nervous system. It infect the CSF, the GIT, the adrenal gland, skin of the neck and even the heart.[10]

CELL WALL

The well - developed cell wall contains a considerable amount of a fatty acid, mycolic acid, covalent attached to the underlying peptidoglycan-bound polysaccharide arabinogalactan, providing an extraordinary lipid barrier. This barrier is responsible for many of the medically challenging physiological characteristics of tuberculosis. The composition and quality of the cell wall components affect the bacteria's virulence and growth rate.

The peptidoglycan polymer confers cell wall rigidity and just external to the bacterial cell membrane, another contributor to the permeability barrier of mycobacteria. The peptidoglycan polymer confers cell wall rigidity and is just external to the bacterial cell membrane, another contributor to the permeability barrier of mycobacteria.

Another important component of the cell wall is lipoarabinomannan, a carbohydrate structural antigen on the outside of the organism that is immunogenic and facilitates the survival of mycobacteria within macrophages. The cell wall is key to the survival of mycobacteria, and a more complete understanding of the biosynthetic pathways and gene functions and the development of antibiotics to prevent formation of the cell wall are areas of great interest.[11]

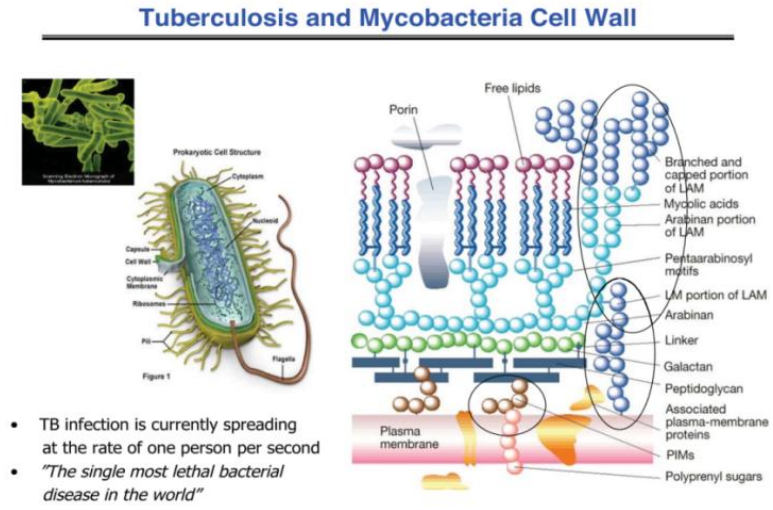

Fig 3: mycobacterial cell wall

Pathogenesis of tuberculosis:

In the lungs, M. tuberculosis is taken up by alveolar macrophages, but they are unable to digest the bacterium. Its cell wall prevents the fusion of the phagosome with a lysosome. Specifically, M. tuberculosis blogs the bridging molecule, early endosomal auto antigen 1(EEA1); however, this blockade does not prevent fusion of vesicle filled with nutrients. Consequently, the bacteria multiply unchecked with in the macrophages. The bacteria also carried the UreC gene, which prevents acidification of the phagosome. The bacteria also evade macrophages-killing by neutralizing reactive nitrogen intermediates. [11]

M. tuberculosis usually enters the alveolar passages of exposed humans in an aerosol droplet, were its first contact is thought be with resident macrophages, but it is also possible that bacteria can be initially ingested by alveolar epithelial type II pneumocystis this cell type is found in greater numbers than macrophages in alveoli, and M. tuberculosis can infect and grow in this pneumocystis ex vivo.[12]

The bacteria are phagocytized in a process that is initiated by bacterial contact with macrophage mannose and/or complement receptors. Surfactant protein A, a glycoprotein found on alveolar surface, can enhance the binding and uptake of M. tuberculosis by up regulating mannose receptor activity.[13]

On the other hand surfactant protein D, similarly located in alveoli, inhibits phagocytosis of M. tuberculosis by blocking mannosyl oligosaccharide residues on the bacterial cell surface, and it is proposed that this prevents M. tuberculosis interaction with mannose receptor on the macrophages cell surface. The human toll-like receptor 2 (TLR2) also plays a role in M. tuberculosis uptake. On entry in to host macrophages, M. tuberculosis resides in an endocytic vacuole called the phagosome. If the normal phagosomal maturation cycle occurs.

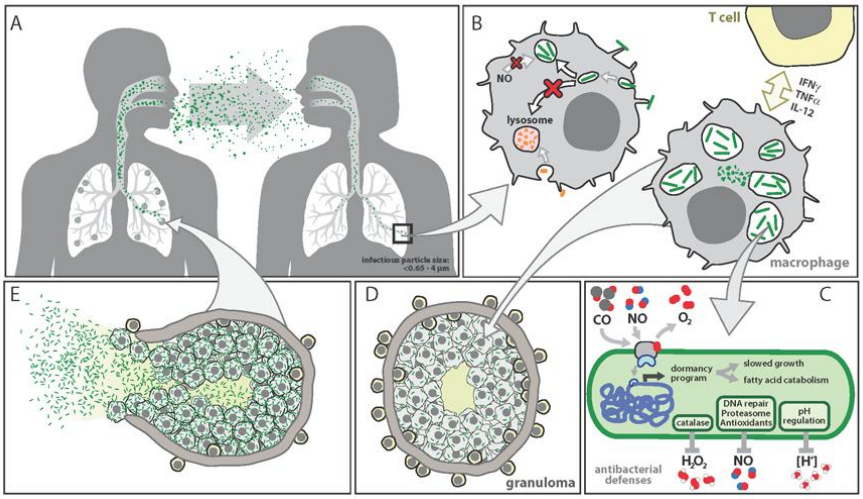

Fig 4: pathogenesis of tuberculosis

Pulmonary

If a tuberculosis infection does become active, it most commonly involves the lungs (in about 90% of cases). Symptoms may include chest pain and a prolonged cough producing sputum. About 25% of people may not have any symptoms (i.e. they remain "asymptomatic"). The upper lung lobes are more frequently affected by tuberculosis than

the lower ones. The reason for this difference is not entirely clear. It may be due either to better air flow, or to poor lymph drainage within the upper lungs. [15]

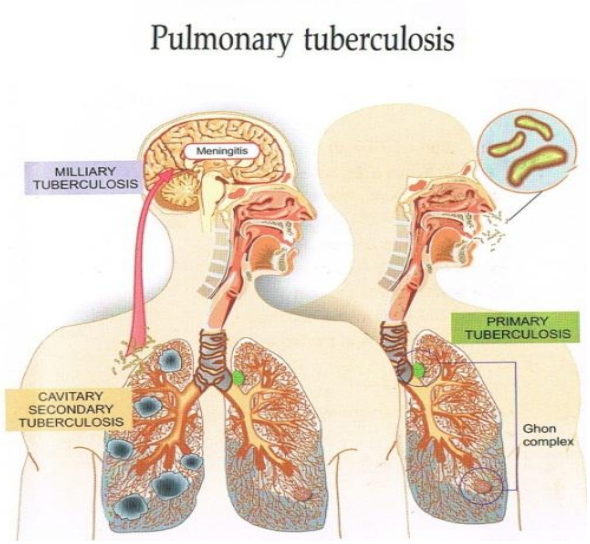

Fig 6: Pulmonary Tuberculosis

Extrapulmonary

In 15–20% of active cases, the infection spreads outside the lungs, causing other kinds of TB. These are collectively denoted as "extrapulmonary tuberculosis". Extrapulmonary TB occurs more commonly in immunosuppressed persons and young children. In those with HIV, this occurs in more than 50% of cases[16]. A potentially more serious, widespread form of TB is called "disseminated" TB, commonly known as miliary tuberculosis. Miliary TB makes up about 10% of extrapulmonary cases.[17]

MECHANISM

Transmission

When people with active pulmonary TB cough, sneeze, speak, sing, or spit, they expel infectious aerosol droplets 0.5 to 5.0 μm in diameter. A single sneeze can release up to 40,000 droplets. Each one of these droplets may transmit the disease, since the infectious dose of tuberculosis is very small (the inhalation of fewer than 10 bacteria may cause an infection). [18]

Transmission should occur from only people with active TB – those with latent infection are not thought to be contagious. The probability of transmission from one person to another depends upon several factors, including the number of infectious droplets expelled by the carrier, the effectiveness of ventilation, the duration of exposure, the virulence of the M. tuberculosis strain, the level of immunity in the uninfected person, and others. [19]

Diagnosis

Diagnosing active tuberculosis based merely on signs and symptoms is difficult, as is diagnosing the disease in those who are immunosuppressed. A diagnosis of TB should, however, be considered in those with signs of lung disease or constitutional symptoms lasting longer than two weeks. A chest X-ray and multiple sputum cultures for acid-fast bacilli are typically part of the initial evaluation. Interferon-γ release assays and tuberculin skin tests are of little use in the developing world. IGRA have similar limitations in those with HIV.[20]leic acid amplification tests and adenosine deaminase testing may allow rapid diagnosis of TB. These tests, however, are not routinely recommended, as they rarely alter how a person is treated. Blood tests to detect antibodies are not specific or sensitive, so they are not recommended

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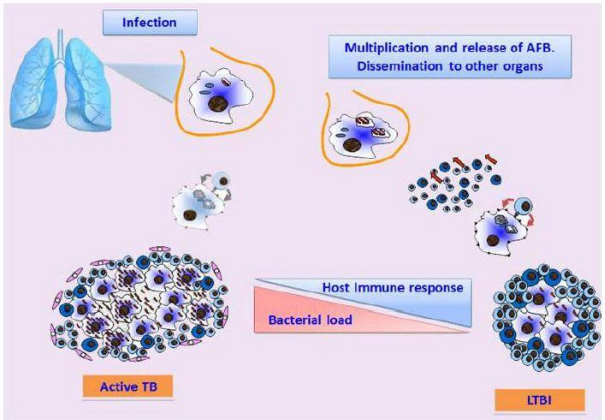


Figure 2 -TB pathogenesis. Tubercle bacilli are inhaled in aerosol droplets, enter into the lungs and, when the host innate immune defenses fail to eliminate the bacteria, *Mtb* start multiplying inside alveolar macrophages and then spreads to other tissues and organs through the bloodstream and lymphatics. Once the cell-mediated immune response kicks in, bacterial replication is usually controlled and in 90-95% of cases non overt signs or symptoms of disease ensue (Latent TB). During latent infection a dynamic equilibrium between the bacilli and host immune responses is established and any event that weakens cell mediated immunity may lead to active bacterial replication, tissue damage and disease occurs (active TB).

Fig 7: Tuberculosis diagnosis

Active tuberculosis

It is spread through the air when people who have an active TB infection cough, sneeze, or otherwise transmit respiratory fluids through the air.

The classic symptoms of active TB infection are a chronic cough with blood-tinged sputum, fever, night sweats, and weight loss (the last of these giving rise to the formerly common term for the disease, "consumption"). Infection of other organs causes a wide range of symptoms. [21] Diagnosis of active TB relies on radiology (commonly chest X-rays), as well as microscopic examination and microbiological culture of body fluids.

Latent tuberculosis

Most infections do not have symptoms, known as latent tuberculosis. About one in ten latent infections eventually progresses to active disease which, if left untreated, kills more than 50% of those so infected.

Fig 8: Latent tuberculosis

Diagnosis of latent TB relies on the tuberculin skin test (TST) and/or blood tests. Treatment is difficult and requires administration of multiple antibiotics over a long period of time. Household, workplace, and social contacts are also screened and treated if necessary. Antibiotic resistance is a growing problem in multiple drug-resistant tuberculosis (MDR-TB) infections. Prevention relies on early detection and treatment of cases and on screening programs and vaccination with the bacillus Calmette-Guérin[22]

Mantoux tuberculin skin test

The Mantoux tuberculin skin test is often used to screen people at high risk for TB. Those who have been previously immunized may have a false-positive test result. Interferon gamma release assays (IGRAs), on a blood sample, are recommended in those who are positive to the Mantoux test. These are not affected by immunization or most environmental mycobacteria, so they generate fewer false-positive results.

Prevention

Tuberculosis prevention and control efforts primarily rely on the vaccination of infants and the detection and appropriate treatment of active cases. The World Health Organization has achieved some success with improved treatment regimens, and a small decrease in case numbers

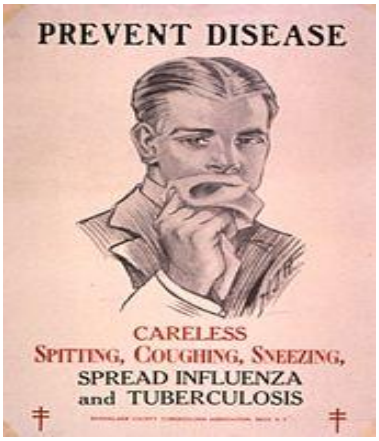


Fig 9: Prevention of disease

Vaccines

The only available vaccine as of 2011 is Bacillus Calmette-Guérin (BCG). In children it decreases the risk of getting the infection by 20% and the risk of infection turning into disease by nearly 60%. It is the most widely used vaccine worldwide, with more than 90% of all children being vaccinated. The immunity it induces decreases after about ten years. A number of new vaccines are currently in development.

Prognosis [23]

Progression from TB infection to overt TB disease occurs when the bacilli overcome the overcome the immune system defenses and begin to multiply. In primary TB disease (some 1–(some 1–5% of cases), this occurs soon after the initial infection. However, in the majority of cases, a latent infection occurs with no obvious symptoms.

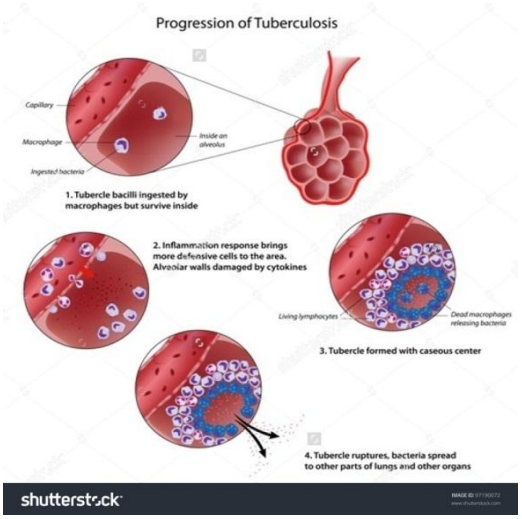


Fig 10: Progression of Tuberculosis

The risk of reactivation increases with immunosuppression, such as that caused by infection with HIV. In people co-infected with M. tuberculosis and HIV, the risk of reactivation increases to 10% per year. Studies using DNA fingerprinting of M. tuberculosis strains have shown reinfection contributes more substantially to recurrent TB than previously thought, with estimates that it might account for more than 50% of reactivated cases in areas where TB is common. The chance of death from a case of tuberculosis is about 4% as of 2008, down from 8% in 1995.

Epidemiology [24][25]

Roughly one-third of the world's population has been infected with M. tuberculosis , with new infections occurring in about 1% of the population each year. In 2010, 8.8 million new cases of TB were diagnosed, and 1.20–1.45 million deaths occurred, most of these occurring in developing countries. Of these 1.45 million deaths, about 0.35 million occur in those also infected with HIV.

Tuberculosis is the second-most common cause of death from infectious disease (after those due to HIV/AIDS). The number of new cases has declined by 17% between 2004–2014. Hopes of totally controlling the disease have been dramatically dampened because of a number of factors, including the difficulty of developing an effective vaccine, the expensive and time-consuming diagnostic process, the necessity of many months of treatment, the increase in HIV-associated tuberculosis. India had the largest total incidence, with an estimated 2.0 million new cases.

The rate of TB varies with age. TB is mainly a disease of older people and the immune compromised (risk factors are listed above). Worldwide, 22 "high-burden" states or countries together experience 80% of cases as well as 83% of deaths.

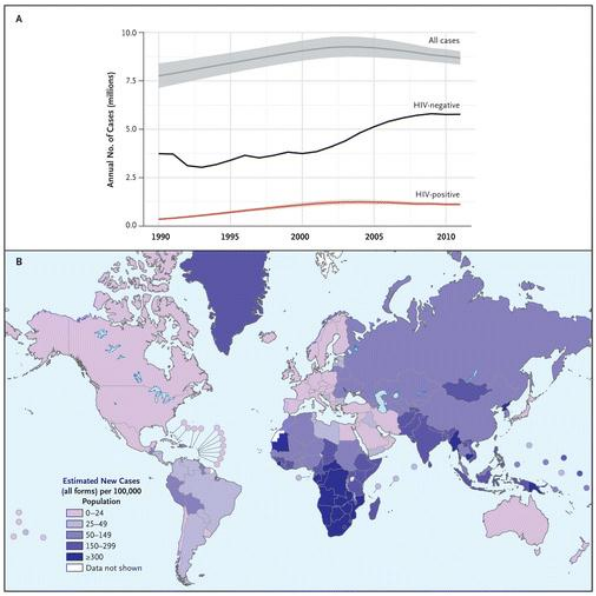


Fig 11: Epidemiology of tuberculosis

GLUTAMINE SYNTHETASE

Glutamine synthetase is an enzyme that plays an essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine:[26]

Glutamate + ATP + NH_3 → Glutamine + ADP + phosphate

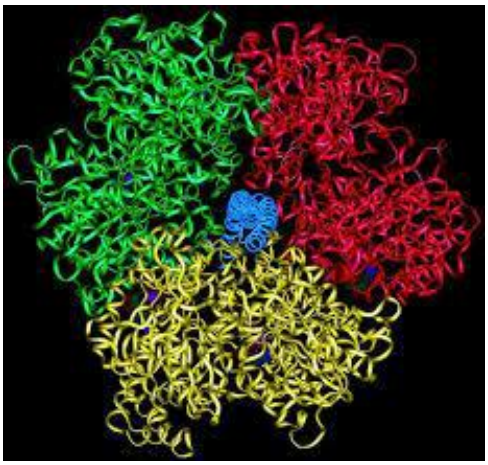
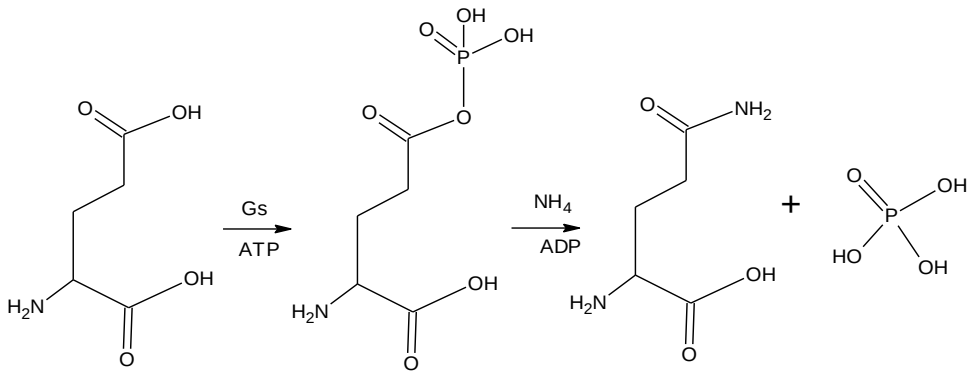


Fig.12. Glutamine synthetase

Glutamine synthetase (GS; EC 6.3.1.2, also known as γ-glutamyl: ammonia Ligase) catalyzes the adenosine 5´-triphosphate (ATP) dependent condensation between glutamate and ammonia, to give glutamine. There are three types of GS, each with a different structure. These enzymes are present in all organisms; eukaryotes express GS type II while prokaryotes mainly express GS types I and III. All GS enzymes have an amino-acid binding site as well as a nucleotide-binding site. They also have three metal ions in the active site between the two pockets, which are necessary for stability and catalytic activity. The amino-acid-binding site is fairly well preserved amongst species, but the nucleotide-binding site differs between mammalian and bacterial enzymes. The focus of the work presented in this thesis was on the GS enzyme of M. tuberculosis.

First, the ATP molecule enters the nucleotide-binding site. Then glutamate enters from the opposite direction into the amino-acid-binding site. The γ-carboxylate of glutamate will then coordinate to either magnesium or manganese ions, and the γ-phosphate of the ATP is transferred to the glutamate. The ammonium ion (NH4+) enters the active site and is deprotonated by an asparagine followed by an attack on the γ-carbonyl. This will release the phosphate (PO43-), and glutamine is formed. [27]



MECHANISM

GS catalyzes the ATP dependent condensation of glutamate with ammonia to yield glutamine. The hydrolysis of ATP drives the first step of a two part, concerted mechanism. ATP phosphorylates glutamate to form ADP and an acyl phosphate intermediate, γ-glutamyl phosphate, which reacts with ammonia, forming glutamine and inorganic phosphate. ADP and Pi do not dissociate until ammonia binds and glutamine is released. ATP binds first to the top of the active site, glutamate binds near the second cation binding site at the bottom of the active site. The presence of ADP causes a conformational shift in GS that stabilizes the γ-glutamyl phosphate moiety. Ammonium binds strongly to GS only if the acylphosphate intermediate is present. Ammonium, rather than ammonia, binds to GS because the binding site is polar and exposed to solvent. In the second step, deprotonation of ammonium allows ammonia to attack the intermediate from its nearby site to form glutamine. Phosphate leaves through the top of the active site, while glutamine leaves through the bottom (between two rings). [28]

NEED TO FOCUS ON TUBERCULOSIS DISEASE

- Tuberculosis is a leading cause of death worldwide**.**

- In the year 2000-2005, there were around 10 to 15 million people with latent TB in the U.S & in 2007, 2.4 million cases were reported.

- In the year 2010, WHO estimated that one-third of the world's population (two billion people) was infected with the bacilli. Of the two billion people, approximately 9.8 million people will develop active tuberculosis and 2.6 million people will die per annum.

- In the year 2013, approximately 13.7 million chronic active cases globally and In the year 2014, approximately 560 thousand new cases per year and 740 thousand new patients infected by both MTB and HIV due to multidrug-resistant Tuberculosis.

NEED FOR NOVEL ANTI-TUBERCULOSIS AGENTS

- A number of once active anti-tuberculosis drugs have now became inactive due to the ever increasing rise in drug resistant strains of tuberculosis. The reason for this is poor patient compliance which leads to the development of more drug resistant strains.

- The lack in patient compliance is due to the fact that the current treatment regimen lasts 6-9 months. The length of the treatment period makes difficulty in killing off the latent and slow-growing bacteria. Drug-resistant TB is a major public health concern.

- The multi -drug resistant TB (MDR-TB) occurs only when drug-susceptible TB is improperly or incompletely treated. According to the World Health Organization(WHO), MDR-TB is defined as a resistance to two of the most effective first line TB agents: Rifampicin and isoniazid. When a strain of TB becomes resistance to any

fluoroquinolone and at least one of the three second-line agents (capreomycin, kanamycin and amikacin), it becomes described at extensively drug-resistant TB(XDR-TB)

- Emergence of Multi-resistant(MDR) strains and high susceptibility of human immunodeficiency virus (HIV) infected persons to the disease forced the scientist to develop novel anti-tuberculosis agents.

- It is evident by these facts; there is an ever growing need to develop novel agents for the treatment of tuberculosis. These new agents should be potent, fast acting, have an excellent pharmacodynamics / pharmacokinetics (PK/PD) profile and have high therapeutic index, and preferably have a novel mechanism of action as to avoid cross-resistant with other agents.

MEDICINAL CHEMISTRY[29]

Medicinal chemistry is the science that deals with the discovery and design of new therapeutic chemicals and their development into useful medicines. Medicinal chemistry may involve synthesis of new molecules, investigation of the relationships between the structure of synthetic compounds and their biological activities, elucidations of their interactions with receptors of various kinds, including enzymes and DNA, the determination of their absorption, transport and distribution properties and studies of the metabolic transformations of these chemicals into other chemicals and their excretion. The drug discovery process involves designing, synthesizing, characterization and evaluation of new chemical entities, suitable for therapeutic use. It also includes study of existing drugs, their biological properties and their quantitative structural activity relationship (QSAR).

DRUG DESIGN[30]

Drug discovery process involves a rapid search for a small molecule often called as lead. A lead molecule is chemical compound which possess pharmacological or biological activity.

Sources of lead compounds can come from natural sources, such as plants, animals, or fungi and also from synthetic chemical libraries.

LEAD OPTIMIZATION[31]

Newly invented pharmacologically active moieties may have poor drug-likeness and may require lead optimization step. This step involves chemical modification of a lead in order to improve their potency, selectively towards binding site, pharmacokinetic parameters and reduced toxicity.

COMPUTER AIDED DRUG DESIGN[32]

The latest breakthroughs in computer-aided drug design, drug delivery systems, and enabling technologies. Computer Aided Drug Design (CADD) and Delivery Systems offers an in-depth discussion of the computer-assisted techniques used to discover, design, and optimize new, effective, and safe drugs.

Drug design, sometimes referred to as rational drug design or simply rational design, is the inventive process of finding new medications based on the knowledge of a biological target. The drug is most commonly an organic small molecule that activates or inhibits the function of a biomolecule such as a protein.

Drug design frequently but not necessarily relies on computer modeling techniques. This type of modeling is often referred to as computer-aided drug design. Finally, drug design that relies on the knowledge of the three-dimensional structure of the biomolecular target is known as structure-based drug design. In addition to small molecules, biopharmaceuticals and especially therapeutic antibodies are an increasingly important class of drugs and computational methods for improving the affinity, selectivity, and stability of these protein-based therapeutics have also been developed.

Furthermore, in vitro experiments complemented with computation methods are increasingly used in early drug discovery to select compounds with more favorable (absorption, distribution, metabolism, and excretion) and toxicological profiles.

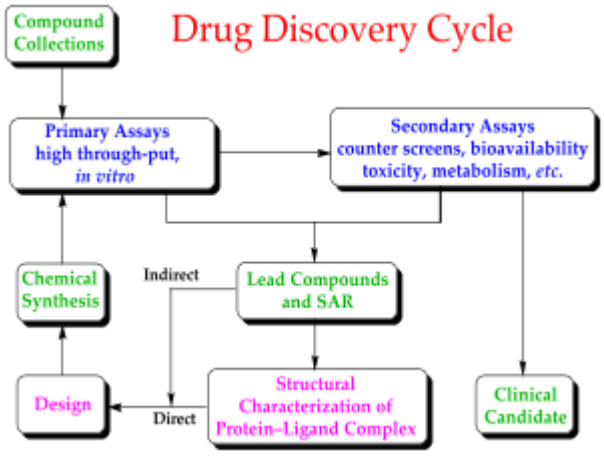


Fig13: drug discovery cycle

Rational drug discovery[33]

In contrast to traditional methods of drug discovery (known as forward pharmacology), which rely on trial-and-error testing of chemical substances on cultured cells or animals, and matching the apparent effects to treatments, rational drug design (also called reverse pharmacology) begins with a hypothesis that modulation of a specific biological target may have therapeutic value.

The search for small molecules that bind to the target is begun by screening libraries of potential drug compounds. This may be done by using the screening assay (a "wet screen"). In addition, if the structure of the target is available, a virtual screen may be performed of candidate drugs. Several methods are available to estimate drug likeness such as Lipinski's Rule of Five and a range of scoring methods such as lipophilic efficiency. Several methods for predicting drug metabolism have also been proposed in the scientific literature.

Drug design with the help of computers may be used at any of the following stages of drug discovery:[34]

1. Hit identification using virtual screening (structure- or ligand-based design)
2. Hit-to-lead optimization of affinity and selectivity (structure-based design, QSAR, etc.)
3. lead optimization of other pharmaceutical properties while maintaining affinity

TYPES [35]

Drug discovery cycle highlighting both ligand-based (indirect) and structure-based (direct) drug design strategies.

There are two major types of drug design. The first is referred to as **ligand-based drug design** and the second, **structure-based drug design**.

Ligand-based

Ligand-based drug design (or **indirect drug design**) relies on knowledge of other molecules that bind to the biological target of interest. These other molecules may be used to derive a pharmacophore model that defines the minimum necessary structural characteristics a molecule must possess in order to bind to the target.

In other words, a model of the biological target may be built based on the knowledge of what binds to it, and this model in turn may be used to design new molecular entities that interact with the target.

Alternatively, a quantitative structure -activity relationship (QSAR), in which a correlation between calculated properties of molecules and their experimentally determined biological activity, may be derived. These QSAR relationships in turn may be used to predict the activity of new analogs.

Structure-based

Structure-based drug design (or **direct drug design**) relies on knowledge of the three dimensional structure of the biological target obtained through methods such as x-ray crystallography or NMR spectroscopy. Using the structure of the biological target, candidate drugs that are predicted to bind with high affinity and selectivity to the target may be designed using interactive graphics and the intuition of a medicinal chemist.

Current methods for structure-based drug design can be divided roughly into three main categories.

The first method is identification of new ligands for a given receptor by searching large databases of 3D structures of small molecules to find those fitting the binding pocket of the receptor using fast approximate docking programs. This method is known as virtual screening.

A second category is de novo design of new ligands. In this method, ligand molecules are built up within the constraints of the binding pocket by assembling small pieces in a stepwise manner. These pieces can be either individual atoms or molecular fragments. The key advantage of such a method is that novel structures, not contained in any database, can be suggested.

A third method is the optimization of known ligands by evaluating proposed analogs within the binding cavity.

Binding site identification

Binding site identification is the first step in structure based design. If the structure of the target or a sufficiently similar homolog is determined in the presence of a bound ligand, then the ligand should be observable in the structure in which case location of the binding site is trivial.

Scoring functions

One early general-purposed empirical scoring function to describe the binding energy of ligands to receptors was developed by Böhm. This empirical scoring function took the form:

Where:

- ΔG_0 – empirically derived offset that in part corresponds to the overall loss of translational and rotational entropy of the ligand upon binding.
- ΔG_{hb} – contribution from hydrogen bonding
- ΔG_{ionic} – contribution from ionic interactions
- ΔG_{lip} – contribution from lipophilic interactions where $|A_{lipo}|$ is surface area of lipophilic contact between the ligand and receptor
- ΔG_{rot} – entropy penalty due to freezing a rotatable in the ligand bond upon binding

A more general thermodynamic "master" equation is as follows:[36]

$$\Delta G_{\text{bind}} = -RT \ln K_{\text{d}}$$

$$K_{\text{d}} = \frac{[\text{Ligand}][\text{Receptor}]}{[\text{Complex}]}$$

$$\Delta G_{\text{bind}} = \Delta G_{\text{desolvation}} + \Delta G_{\text{motion}} + \Delta G_{\text{configuration}} + \Delta G_{\text{interaction}}$$

Where:

- desolvation – enthalpic penalty for removing the ligand from solvent
- motion – entropic penalty for reducing the degrees of freedom when a ligand binds to its receptor

- configuration – conformational strain energy required to put the ligand in its "active" conformation

- interaction – enthalpic gain for "resolvating" the ligand with its receptor

- According to Gibbs free energy equation, the relation between dissociation equilibrium constant, K_d, and the components of free energy was built.

The divergent development of tuberculosis as a disease means that easily accessible and effective diagnostic tools are extremely important in identifying possible transmission vectors as well as funneling treatment to the populations in most dire need.

IN-SILICO DRUG DESIGN

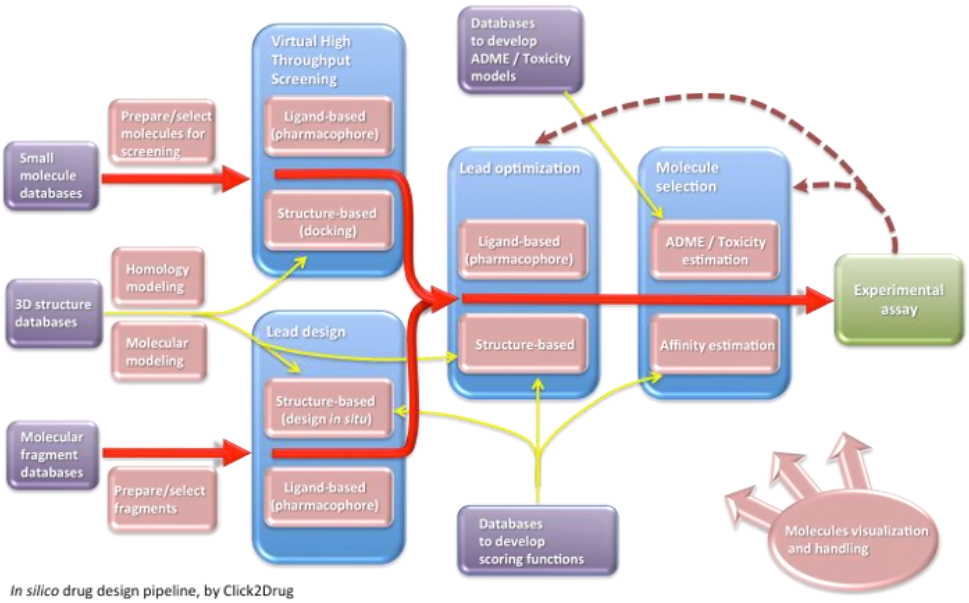


Fig 14: In-silico drug design

DOCKING

Docking program is used to fit the ligand molecule into the target structure in a variety of position, conformations and orientations. Docking mode is known as pose. Each pose scored based on its complementarity to the target in terms of shape and properties such as electrostatics in order to identify the most favorable energetical pose.

The quality of any docking results depends on the starting structure of both the protein and the potential ligand. The protein and ligand structure need to be prepared to achieve the best docking results.

PROTEIN PREPARATION

It is now accepted that the old idea of the "key and lock" interaction of a ligand and its protein. Receptor is not an accurate description of most biological complexes. The ligand-protein interactions resemble more a "hand and glove" association, where both parts are flexible and adjust to complement each other induced fit. The can modify their shape and mould their complementarity. So, as to increase favorable contacts and reduced adverse interactions, maximizing the total binding free energy. It has been found that active site regions of enzymes appear to present areas of both low and high conformational stability.

RECEPTOR CONFORMATION

` The three dimensional (3-D) structures of both ligand and protein are necessary for the application of docking techniques. While the manifold of conformational structures of small molecule may be relatively easy to predict, the lowest energy conformation obtained may not correspond to that of the bound ligand.

Many proteins targeted for drug design do not have an experimentally determined structure and, therefore, docking studied cannot be performed directly. In some cases, computational techniques can be used to predict the 3D structure of a protein provided the structure of a closely related protein homolog is known. Homology modeling or sequence threading techniques may be used to generate models of protein structure which, although not as good as experimentally determined structures, can be used as docking targets.

AIM AND OBJECTIVE

AIM

The aim of this project is to design molecules into potential anti-tubercular activity that is capable of inhibiting cell wall synthesis by inhibiting glutamine synthetase. The designed compounds will be synthesized, characterized and evaluated for activity and toxicity.

OBJECTIVE

The compounds are designed and docked against a specific crucial target, Glutamine Synthetase I, which is involved in the cell wall biosynthesis and nitrogen metabolism. The synthesized compounds are expected to act on the same.

DESIGN

In-silico design of glutamine synthetase 1 inhibitors.

SYNTHESIS

A novel anti-tubercular activity containing compounds are synthesized under specific conditions, which is effective against specific microbes.

CHARACTERIZATION

The above synthesized compounds will be identified and characterized by following methods

- Melting point
- TLC method
- Infrared Spectroscopy
- Nuclear Magnetic Resonance
- Mass spectroscopy
- Gas Chromatography and Mass Spectroscopy (GC-MS) and if necessary
- Liquid Chromatography and Mass Spectroscopy (LC-MS)

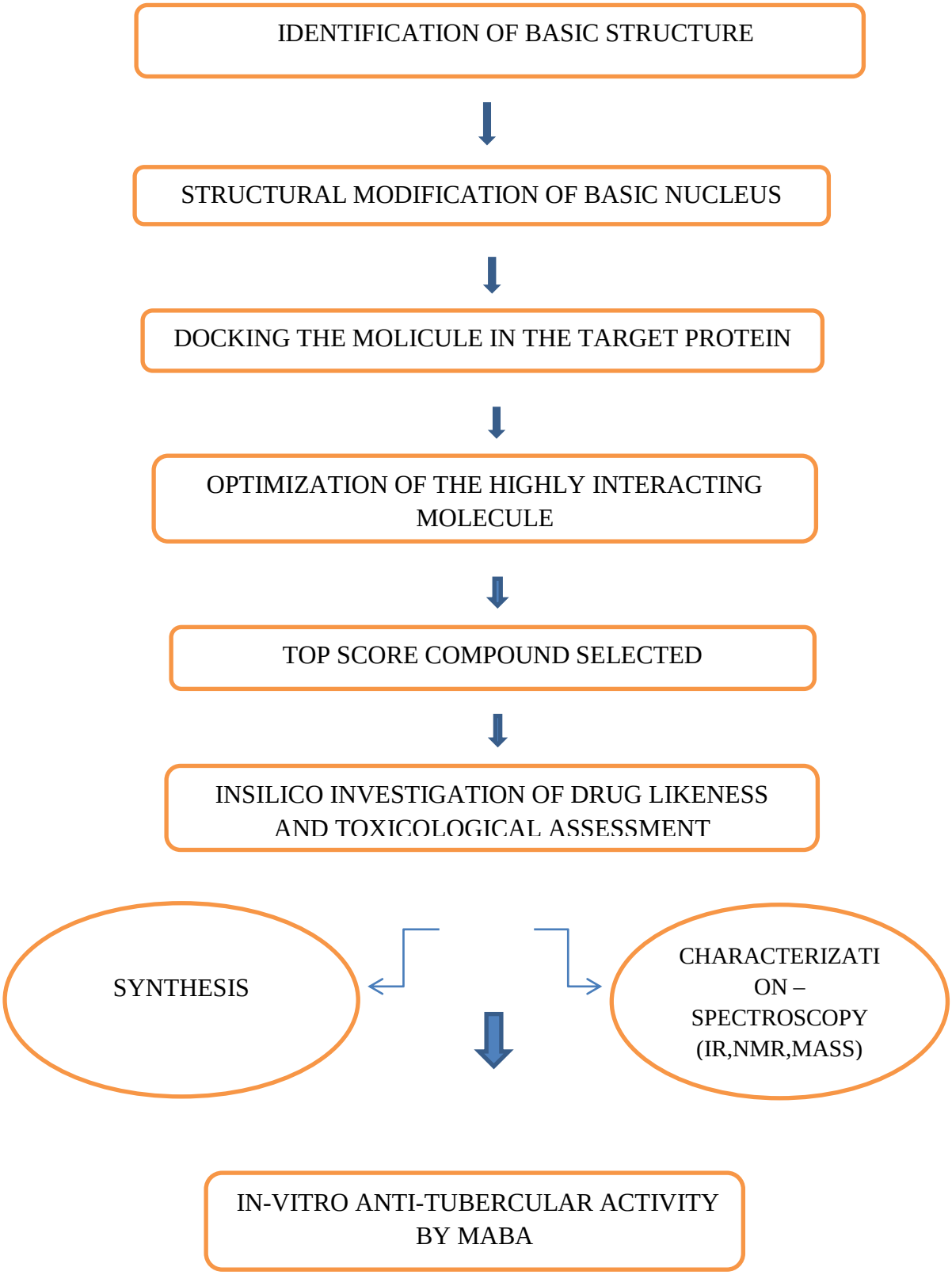
BIOLOGICAL EVALUATION

The synthesized compounds will be screened for their anti-tubercular activity by in-vitro methods.

TOXICOLOGICAL PREDICTION

Toxicological prediction will be carried out for synthesized compounds by in-silico property explorer like OSIRIS.

The present study will be conducted according to the following design



PLAN OF WORK

- The main aim of this project is to synthesize a novel compounds containing anti-tubercular activity but my plan of work is over a 1000 molecules, a desired anti-tubercular activity containing molecule is selected and an enzyme which is active against tubercular cells.

- The selected enzyme is docking against compounds which is to be synthesized, in this docking score; drug interaction; binding action is important

- The selected compounds were synthesized at appropriate manner then purified by recrystallization, characterized by sharp melting point, TLC.

- The following compounds are confirmed by characterized techniques which is hyphenated like, GC-MS and LC-MS analysis(if necessary).In this technique, molecular weight and purity of the formed compounds are determined

- The molecular structure of the compounds are interpretate by proton NMR (1HNMR) and the functional groups of the compounds are analyzed by IR studies

- The anti-tubercular activity of the compounds are evaluated by MABA

- The toxicity of synthesized compounds are visualized by OSIRIS

Finally, a novel and potent anti-tubercular activity containing compounds are synthesized, purified, characterized and evaluated

REVIEW OF LITERATURE

On the basis of the designed process, thiophene have been identified as potential Anti-tubercular molecules. It was therefore decided to conduct a literature survey of thiophene moieties and of the target glutamine synthetase

1.Reviews related to the target- Glutamine synthase

Marcus A. horwitz *et al.,* [39] assessed the role of glutamine synthetase (GS), in the pathogenicity of mycobacterium tuberculosis; glnA1 was constructed via allelic exchange. The mutant had no detectable GS protein or GS activity and was auxotrophic for L-glutamine. In addition, the mutant was attenuated for intracellular growth in human THP-1 macrophages. Based on growth rates of the mutant in the presence of various concentrations of L-glutamine the importance of the enzyme was known. These studies demonstrate that glnA1 is essential for M.tuberculosis virulence.

Berlicki L; kafarski [40] studied about the Glutamine synthases enzyme which catalyses the formation of glutamine from glutamine and ammonium ion. It is one of the most important enzymes in nitrogen metabolism. The first part of the review presents the long-dating research on inhibitors of glutamine synthetase. Analysis of their structure activity relationship is presented in some detail. The second part of the paper is dedicated to potential medical applications of glutamine synthetase inhibitors, which is proved as effective anti-tuberculosis agent with high selectivity towards the pathogen.

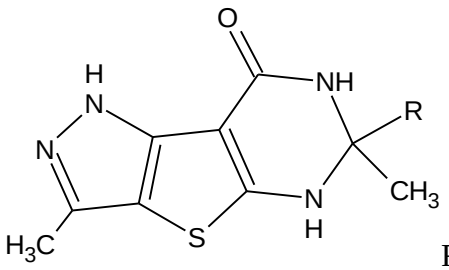
Oolf Lagerlund[41] synthesized some potential anti-tubercular agents which targeted Glutamine Synthetase (GS), is one of the latest targets of M.tb which catalyses the formation of glutamine from glutamic acid. In this work, novel GS inhibitors and new Pd (0) - catalyzed methods have been developed.

Wojciech W. krajewski *et al.*,[42] summarized that glutamine synthetase catalyzes the ligation of glutamate and ammonia to form glutamine, with the hydrolysis of ATP. The enzyme is a central component of bacterial nitrogen metabolism and is a potential drug target. This study provides the first reported structure for a tauto form of the tuberculosis enzyme.

The phospho compound, generated in situ by an active enzyme, mimics the phosphorylated tetrahedral adduct at the transition state. Some differences in ligand interactions of the protein with phosphorylated compound and nucleotide are observed compared with the earlier structures; a third metal ion also is found.

B) **Of the several works of thiophene, few of them are enlisted here in support of their thiophene antitubercular activity**

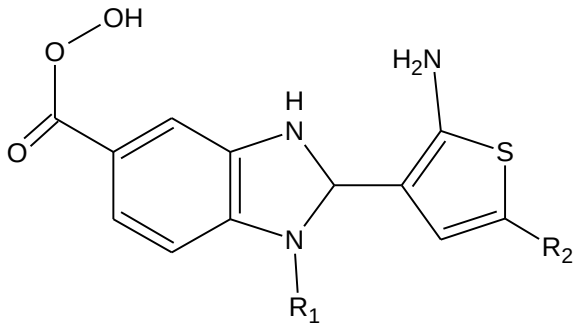
Shailesh J. Vaghasiya.et.al **[43]** evaluated synthesis, biological screening of novel pyrozolo (3',4';4,5) thieno (2,3-d) pyrimidin-8-ones, it was involved in a new synthetic route is proposed for the synthesis of 3,6-dimethyl-6-aryl- 1,5,6,7-tetra hydro-8H-pyrozolo(3',4':4,5)thieno (2,3-d) pyrimidin 8-one from 5-amino-3-methyl-1H-thieno(3,2c)pyrozole 6-carbanonitrile. The compounds **(3a-h)** were initially screened against *Mycobacterium tuberculosis* H_{37} *RV* (ATCC 27294) (American Type Culture Collection, Manassas, Va.) at the single concentration of 6.25 µg/ml in BACTEC 12B medium using a broth micro dilution assay, the microplate Alamar blue assay (MABA)



R – Phenyl; 2-OH phenyl

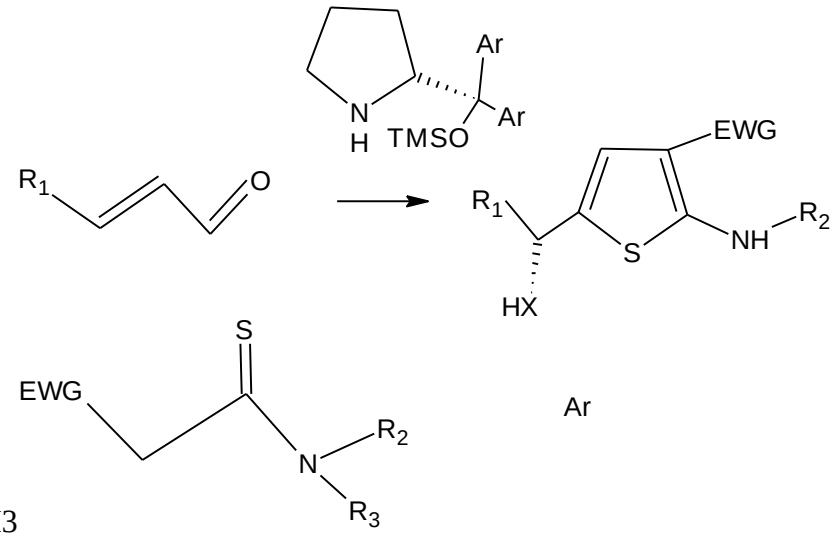
3**a-h**

Li-Hsun chen et.al:[44] described utilizing 2-cyano methyl benzimidazole in a modified Gewald multicomponent reaction. The synthesis strategy involved treatment of 2(cyanomethyl)-benzimidazole with aldehydes containing an active methylene group and sulfur powder under refluxing conditions. achieved a multicomponent condensation reaction on an ionic liquid support to afford the benzimidazole linked thiophene.



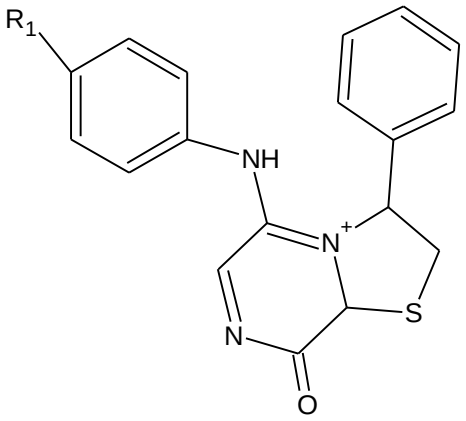
C.Oliver kappe:[45] reported that synthesized some novel thiophene derivatives by microwave assisted methods, using gewald reaction it has been described anti-microbial activity; anti-tubercular activity

Ransborg.L.K.et.al[46]**:** revealed that the synthesis and anti-mycobacterial evaluation of some optically active thiophenes via an organometallic one-pot methodology



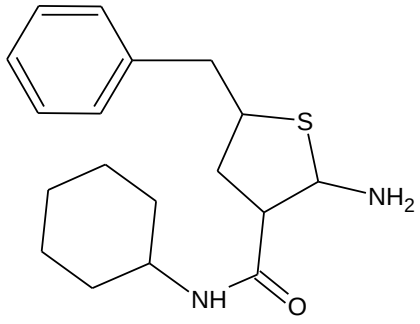
Ar: 3,5-(CF3)2-C6H3

Dong Cai 2015[47]: The compounds were evaluated for their preliminary *in vitro* antibacterial activity against some Gram-positive and Gram-negative bacteria and screened for antitubercular activity against *Mycobacterium tuberculosis* by the broth dilution assay method. Some compounds showed good antibacterial and antitubercular activities. The structures of the target compounds were confirmed by IR, 1H-NMR, 13C-NMR and HRMS studies.

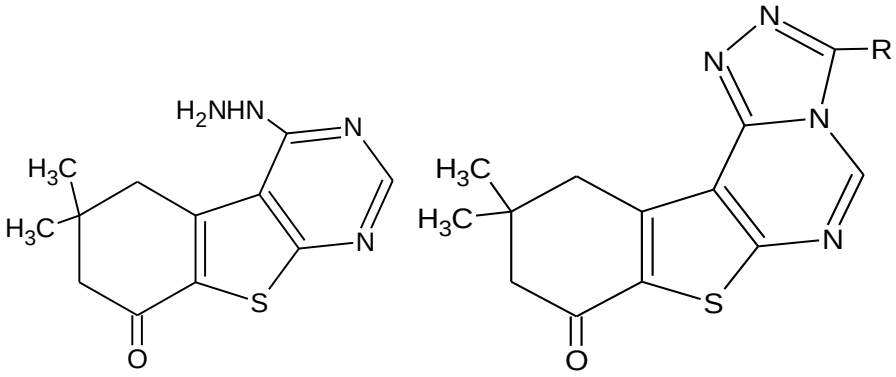


Kan Wang.et.al[48]: describe valuable general protocols for the synthesis of arrays of 2-aminothiophene-3-carboxamides from cyanoacetamides, aldehydes or ketones and sulfur via a Gewald-3CR variation. In many cases the reactions involve a very convenient work up by simple

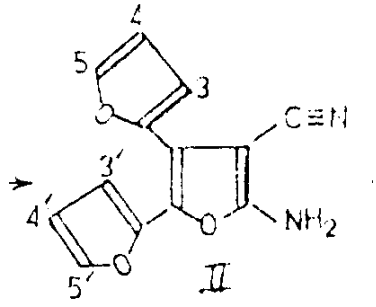
Precipitation in water and filtration. >40 new products are described.



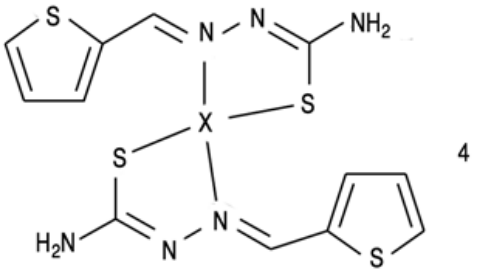
Nitinkumar S. Shetty (2014)[49]: In this journal, said that a facile microwave-assisted synthesis of novel thienopyrimidines and triazolothienopyrimidines and their anti-microbial activities. The following compounds showed promising anti-bacterial activity and anti-fungal activity. Biological screening of these compounds shows mycobacterial activity; those activity was 62% of that of ampicillin against B. subtilis.



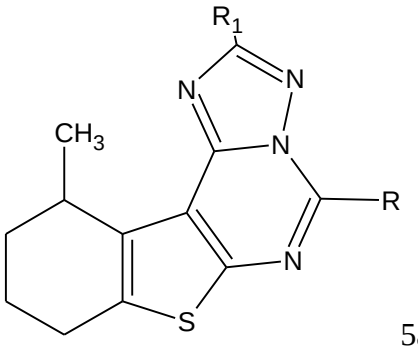
Vedia Ertuzun (1986[50]): studies described substituted heteroaromatic amines were synthesized by a cyclization reaction achieved by means a special base catalysed cyclization reaction, using alpha-hydroxy ketone and active methylene compounds. The purpose of this study was to synthesize substituted 2-amino-furane by placing furyl groups to the 4- and 5-positions of the substituted heteroaromatic ring, using a special Knoevenagel condensation reaction; The pKa value of the compound was determined as 18.0, this reveals that furyl groups have much more electron withdrawing effect than phenyl groups.



Amir R. Jalilian.et.al (2008)[51]: This study shows a review of preparation and evaluation of isotopic thiophene-2-aldehyde thiosemicarbazide PET studies. Total labelling and formulation of [61Cu]TATS took about 40minutes, with a yield of more than 90%. A significant specific activity (9.1 TBq/mmol or 246 Ci/mmol) was formed via insertion of 61Cu cations.

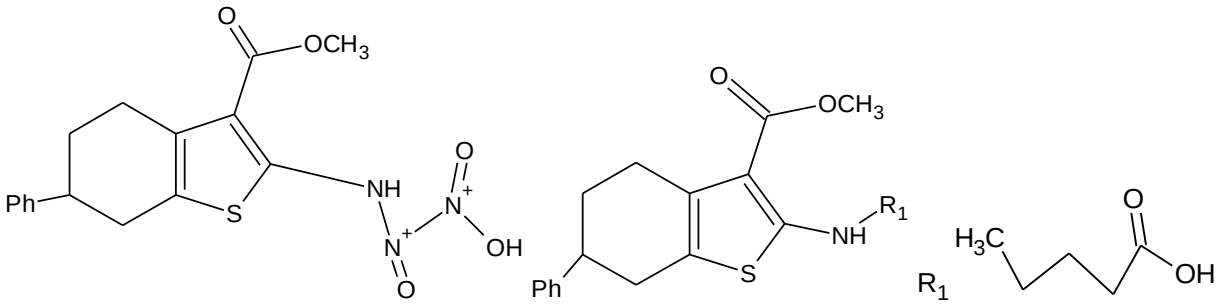


AshrafY.khan.et.al[52]: reported that the synthesis, characterization and biological evaluation of novel thienopyrimidine and triazolotheinopyrimidine derivatives as anti-tubercular and anti-bacterial agents by gewald reaction. Most of these compounds exhibited MIC values in the range of 20 – 100 μM against *Mycobacterium tuberculosis H37Rv*. In the series, compound **5c** was most active with MIC 20 μM. Some of these compounds exhibited MIC values in the range 8 - 64μM. Compound 5c was found to be the most active with an MIC of 5 and 8μM respectively.

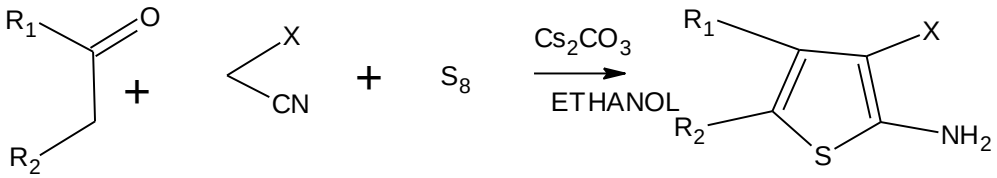


5a-c, R=CH3, R1=H, CH3, CH2CH3

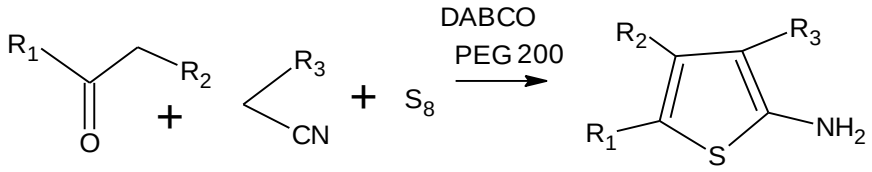
Lianpao Wu[53]: it evaluated a high throughput screening of 17,500 compounds, two hit compounds containing a tetrahydrobenzothiophene core were identified as interesting biofilm inhibitors of *Escherichia Coli* UTI89. Thirteen compounds have been successfully synthesized from methyl 2-amino-6-phenyl-4, 5, 6, 7-tetrahydrobenzo[*b*] thiophene-3-carboxylate, which was prepared from Gewald three-component reaction.



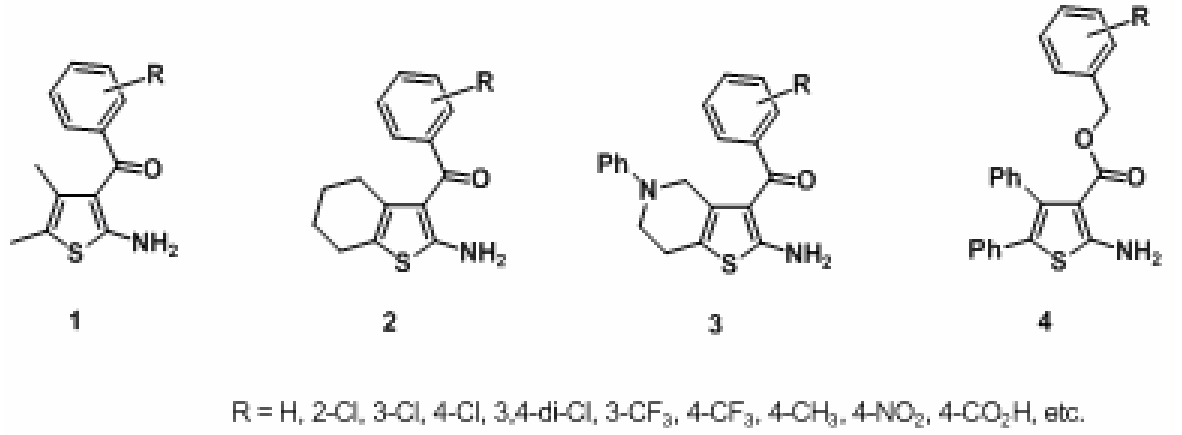
Farid Moeinpour.et.al(2011):[54] It describes that Cesium Carbonate as a Heterogeneous Base Catalyst for Synthesis of 2-Aminothiophenes via Gewald Reaction. The synthesis of substituted 2-aminothiophenes is attractive to chemical researchers as they are important intermediates in organic synthesis and frequently used as the scaffold motif of a variety of agrochemicals, dyes, and biologically active products.



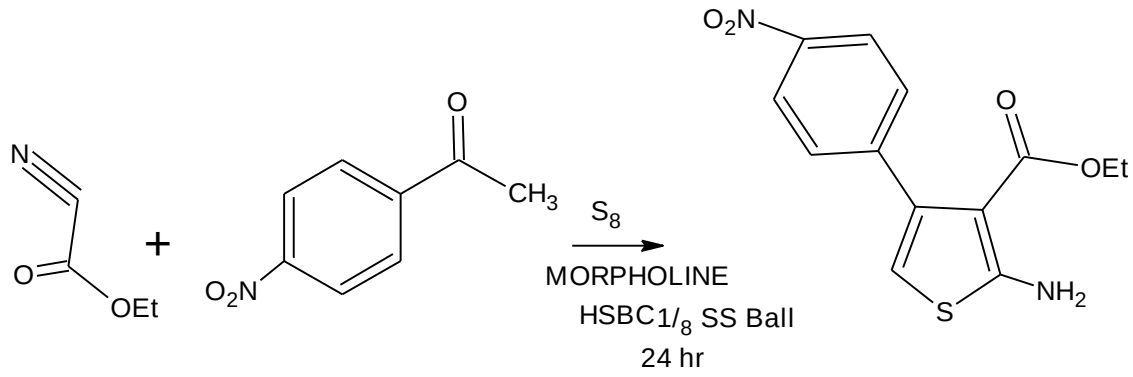
Chengyuan Liang.et.al (2014):[55] It reveals that to describe Ultrasound-promoted synthesis of 2-aminothiophenes accelerated by DABCO utilizing PEG-200 as solvent. An expeditious and greener one-pot procedure was developed for the synthesis of multisubstituted 2-aminothiophene derivatives. In the presence of catalytic amount of DABCO, ketones or aldehydes, dicyanomethane and elemental sulfur were converted into the corresponding 2-aminothiophene derivatives in moderate to high yields in PEG-200 under sonication.



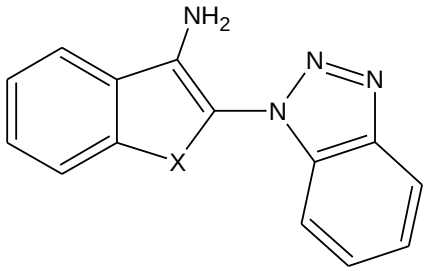
Zita Puterova.et.al(2009):[56] It involved in application of substituted 2-amino thiophene in drug design. Highly substituted thiophene derivatives are important heterocycles found in numerous biologically active compounds.



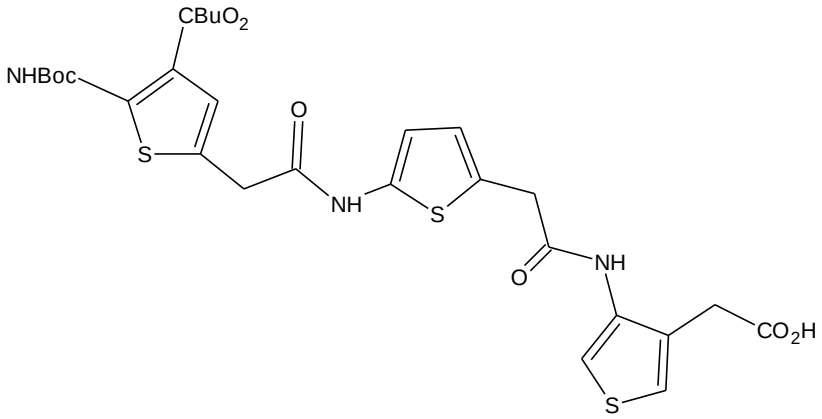
William C. Shearouse (2014):[57] This studies show a solvent-free, one-step, one-pot gewald reaction for alkyl-aryl ketones via mechanochemistry, the Gewald reaction can be catalytic in base, and conducted under aerobic conditions. Using thermal heat in tandem with the mixer/mill significantly increases the rate of reaction.



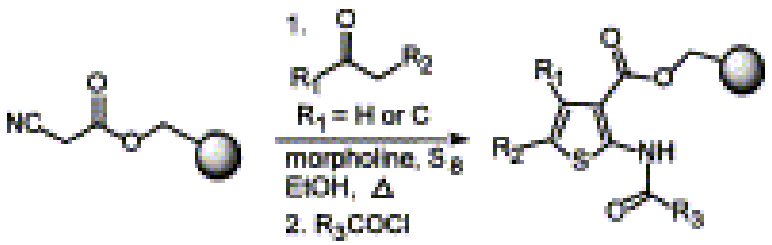
Stanislav Radl (2005):[58] it involved in the modification of the Gewald methodology for the synthesis of 3-amino-2-(1H-1,2,3-benzotriazol-1-yl) substituted benzofurans, benzothiophenes and 1H-indoles. The synthesized compound shows promising anti-bacterial activity which was evaluated by MABA.



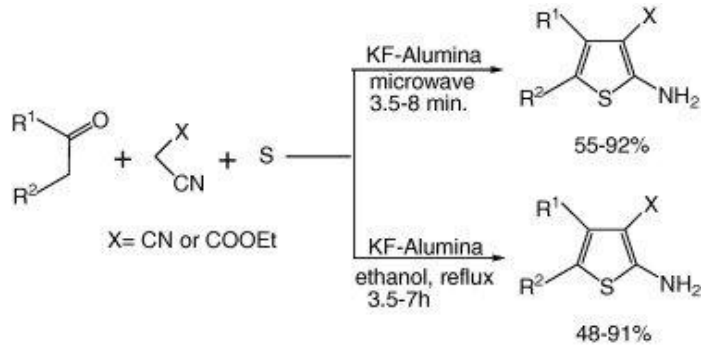
Gewald thiophene synthesis with siloxycylopropanes (1961) [59]: It involved in the synthesis of polysubstituted thiophenes from the multicomponent condensation of ketones or aldehydes, activated nitriles and elemental sulfur was originally published in 1961 by Gewald and co-workers.



Castanedo.G.M:[60] This studies reveals that the synthesis of tetra substituted thiophene on solid-support using the Gewald reaction. This reaction combines a ketone or aldehyde, an activated nitrile,and sulfur in the presence of a suitable amine base to make tri and tetra substituted thiophenes. After optimizing conditions for maximum yield and purity, the scope of the reaction was investigated. Finally this method is highlight in the synthesis of two biologically relevant compounds



Sridhar.M.et.al:[61] In this study, reaction assisted by microwave accelerated Gewald reaction involved in the synthesis of 2-aminothiophene by multicomponent reaction of a ketone with an active nitrile and elemental sulfur under KF-alumina catalysis is described



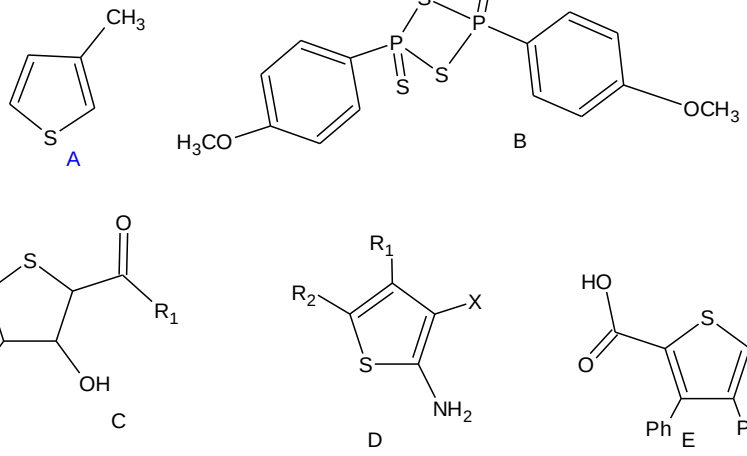
Hallas. G[62] it states that, dyes derived from aminothiophene involved in the synthesis of some heterocyclic disperse dyes using the Gewald reaction. A series of red to violet thienyl-2-azo disperse dyes has been derived from aminothiophenes synthesised directly by using the Gewald reaction. 13C NMR data for certain derivatives are reported.

Synthetic communications (International Journal)[63]: describes organic reactions in ionic liquids, Gewald synthesis of 2-aminothiophenes catalyzed by Ethylenediammonium Diacetate. Ionic liquids based on 1-butyl-3-methylimidazolium tetrafluoroborate ($BmimBF_4$) and 1-butyl-3-methylimidazolium hexafluorophosphate ($BmimPF_6$) were used as reusable alternatives to volatile organic solvents (VOCs) for ethylenediammonium diacetate (EDDA) catalyzed Gewald synthesis of 2-aminothiophenes. Significant rate enhancement and improvement of the yield were observed. The ionic liquids containing catalyst EDDA were recycled several times with no decreases in yields and reaction rates.

REVIEW RELATED TO THIOPHENE NUCLEUS AND ITS BIOLOGICAL ACTIVITIES

Mishra.R[64]: It involved in review of synthesis, properties and biological activity of thiophene nucleus has been established as the potential entity in the largely growing chemical world of heterocyclic compounds possessing promising pharmacological characteristics. The recent review provides a broad view of the synthesis and properties of compounds having thiophene nucleus.

Well known major synthetic procedure includes: paal-knorr thiophene synthesis, fiesselmann thiophene synthesis, Gewald aminothiophene synthesis and hinsberg synthesis.

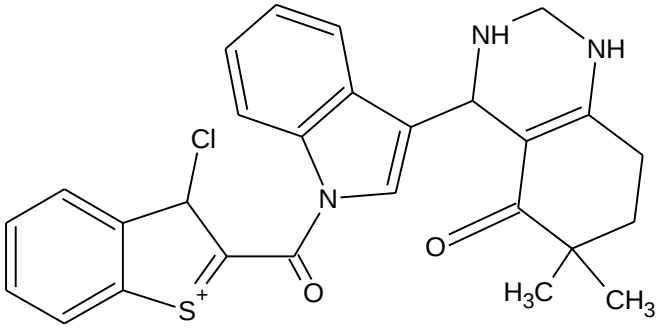


Rajeev kharb[65]**:** reported that to design and synthesize novel antimicrobial agents to solve the problem of microbial resistance towards conventional antimicrobial agents. Among the various types of heterocyclic compounds, benzothiophene plays an important role in the medicinal chemistry because it possesses promising antibacterial, antifungal and antitubercular activities.

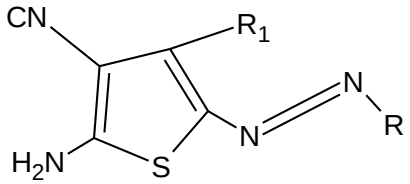
This articles aims to review antimicrobial activities of novel benzothiophene derivatives during recent years which reveal their biological potential as anti-infective agents.

Bhatt.R.et.al:[66] studies reveals that the synthesis, characterization and anti-microbial evaluation of some tetrahydroquinazoline derivatives of benzothiophenes.

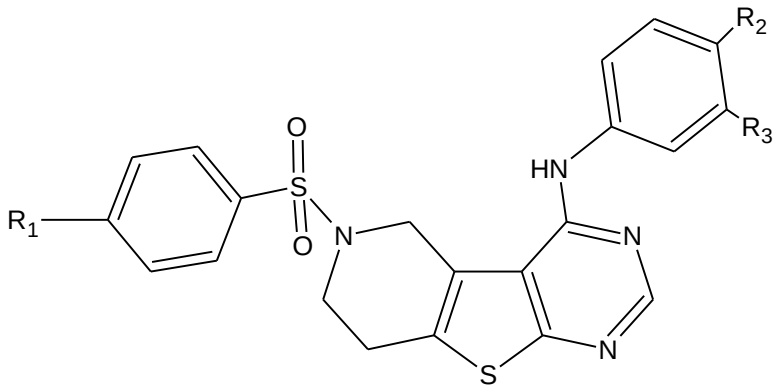
The structures of the newly synthesized compounds were elucidated on the basis of IR and 1H NMR spectral data of the compounds.



Abdou.M.M.et.al[67]: thiophene nucleus may also be used in the field of dyes chemistry. A series of red to blue hetarylazo dyes **15** with good fastness was synthesized by coupling reaction of 4-aryl-2-aminothiophenes derivatives with diazotized 5-nitro-2-aminothiazole and 6-nitro-2-aminobenzothiazole, respectively, as diazo components.

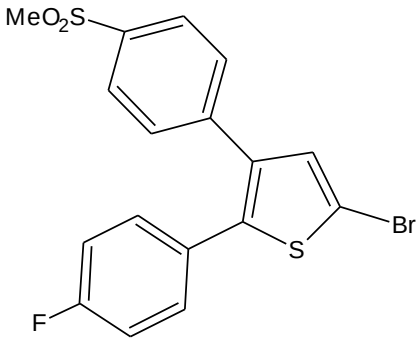


Mittal.M.et.al:[68] synthesis, characterization and anti-microbial activity of substituted tricyclic compounds, it may be 5,6,7,8-tetrahydro pyrido[4',3':4,5]thieno[2,3-D] pyrimidines using piperidin-4-one Hydrochloride and benzenesulfonyl chloride asthe starting material. All the synthesized products were evaluated for their antibacterial activity. The structures of all the synthesized compounds have been determined by their spectral and microanalytical data.



Kallepalli.V.A.et.al:[69] it is a divergent synthesis; which involved in the synthesis of 2,3,5-substituted thiophenes by C-H activation/borylation/Suzuki coupling.

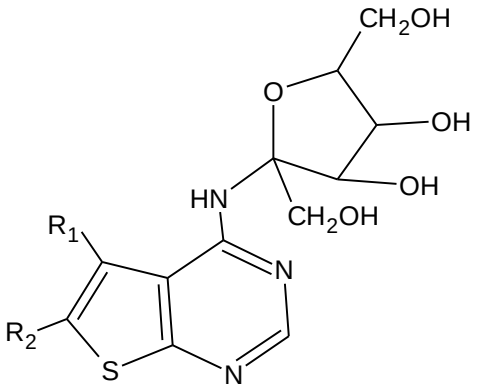
2 Thiophenes are an important class of heterocyclic compounds with applications in the design of advanced materials to the treatment of various diseases.



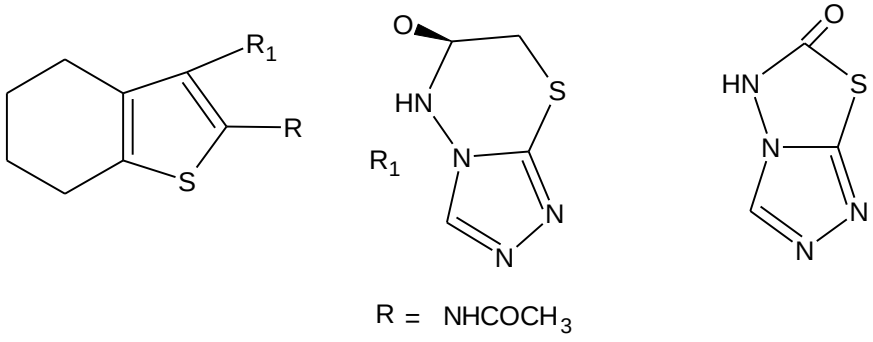
Sidhu.P.S.et.al(2014):[70] it is a computational studies that involved in the QSAR study of thiophene-anthranilamides based factor Xa direct inhibitors.

QSAR studies were performed to understand the structure activity relationship (SAR) and to build the computational model to predict newer inhibitors with improved potency. In this study, a library of thiophene-anthranilamide based inhibitors of factor Xa was used to develop QSAR model. The library was divided into two sets: Training and Test sets. QSAR Model consists of four descriptors with R-square value of 0.80. Based on the statistical parameters, this model can be used to predict the newer inhibitors with improved pharmacological profile.

Verma.A.K.et.al:[71] it involved in the synthesis, characterization and screened for anti-mycobacterial activity of novel N-(sugar pyranosyl) thienopyrimidine 4-amine derivatives. These derivatives were identified on the basis of melting point range, Rf values, IR and 1H NMR spectral analysis. The derivatives exhibited significant to promising antimicrobial activity

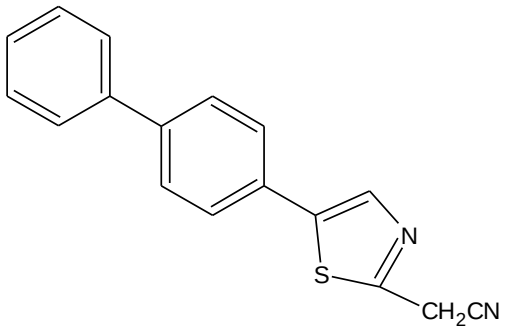


Chaudhary.A.et.al (2012)[72]: describes biological diversity of thiophene, it explained about review of thiophene. Thiophene has been established as the potential entity in the largely growing chemical world of heterocyclic compounds possessing promising pharmacological characteristics. This review provides various synthetic strategies of thiophene analogues. The following compounds provides promising anti-microbial activity.

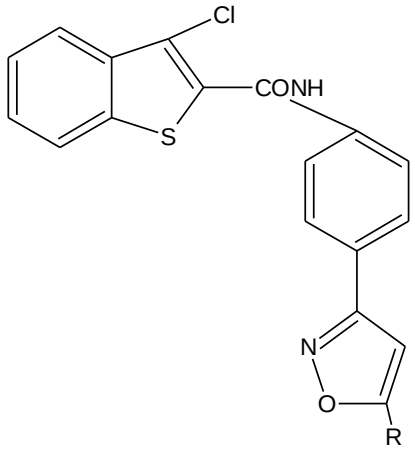


Deligeorgiev.T.et.al (2011)[73]: concluded an environmentally benign synthesis of 2-cyanomethyl-4-phenylthiazoles under focused microwave irradiation. The methods allows the

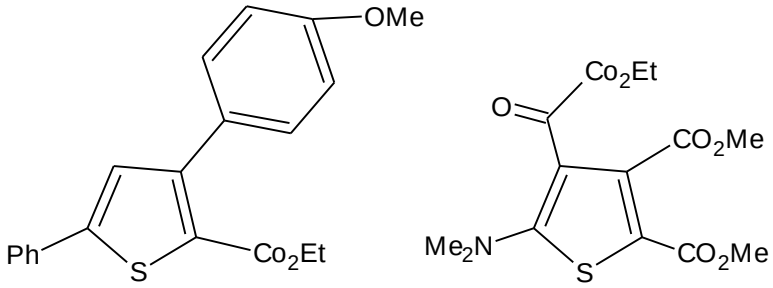
synthesis of products in excellent yields with short times and the work-up is easy. This approach can be applied to the preparation of a variety of derivatives.



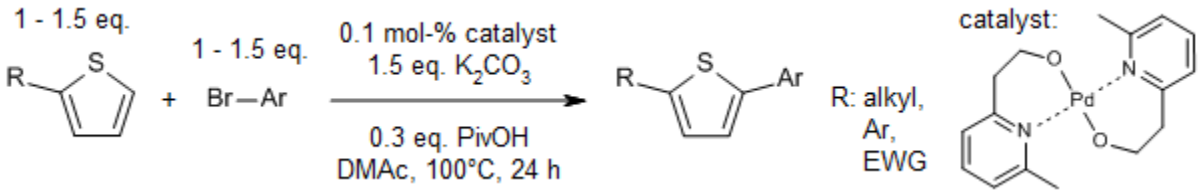
Kachhadia.V.V.et.al (2004):[74] described synthesis of isoxazoles and cyanopyridines bearing benzothiophenes nucleus as potential anti-tubercular and anti-microbial agents. The reaction was carried out by condensing chalcones with hydroxylamine hydrochloride and malononitrile. All the synthesized compounds were established on the basis of elemental analyses IR, 1H NMR and Mass spectral data and screened for their anti-tubercular and anti-microbial activities.



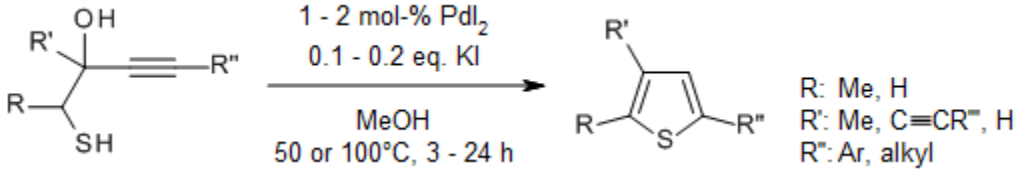
Mancuso.R.et.al (2014):[75] described review of recent advances in the synthesis of thiophene derivatives by cyclization of fuctionalized alkynes.



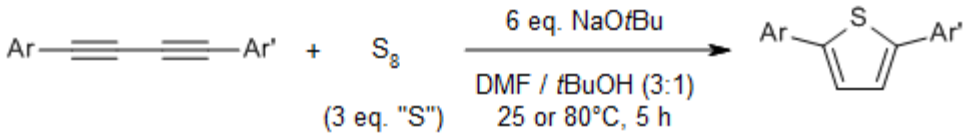
Y. Li, J. Wang.et.al (2014):[76] described low catalyst loading of a bis(alkoxo)palladium complex enables an efficient phosphine-free direct C-H arylation of thiophenes at C2. The developed synthetic method couples aryl or heteroaryl bromides with thiophenes bearing electron-donating or electron-withdrawing groups and other heterocyclic moieties such as benzothiophenes.



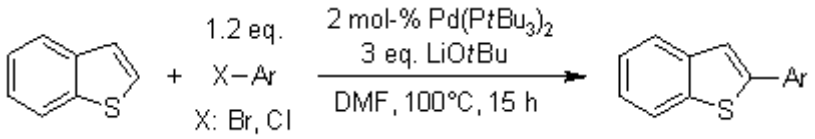
B. Gabriele.et.al:[77] Various readily available 1-mercapto-3-yn-2-ols 5 were conveniently converted into the corresponding thiophenes in good to high yields in MeOH as solvent in the presence of PdI_2 as catalyst and KI as additive. The use of $BmimBF_4$ as solvent enables the recycling of the catalyst.



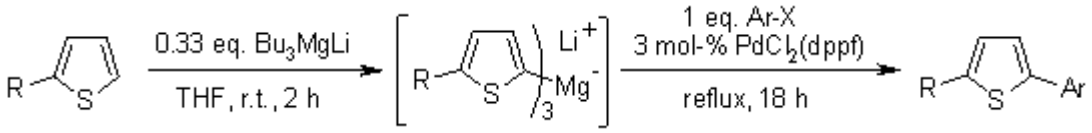
G. Zhang.et.al:[78] The interaction between elemental sulfur and NaO*t*Bu enables a facile single-step protocol for the synthesis of thiophene derivatives from 1,3-diynes. A plausible mechanism based on EPR experiments revealed that the trisulfur radical anion acts as a key intermediate of this process.



S. Tamba.et.al:[79] A palladium-catalyzed C-H arylation of electron-enriched heteroarenes with aryl bromides and aryl chlorides proceeds in the presence of LiO-*t*-Bu as a base.



O. Bayh.et.al:[80] Thiophene was regioselectively deprotonated by treatment with Bu_3MgLi in THF at room temperature. The lithium arylmagnesate formed was either trapped with electrophiles or cross-coupled in a 'one-pot' procedure with aryl halides under palladium catalysis.



4.Reviews related to biological evaluation of antitubercular activity by MABA

Sephra N.Ramprasad [81] studied the various applications of Alamar blue as an indicator. Alamar blue is an indicator that is used to evaluated metabolic function and cellular health. The Alamar blue bioassay is being utilized to access cell viability and cytotoxicity in a biological and environmental system and in a number of cell types including bacteria, yeast, fungi, and protozoa.

Jose d Jesus Alba-Romero et al [82] applied the Alamar blue assay to determine the susceptibility to anti-tuberculosis pharmaceuticals. The results showed that the MABA test is fast and easy to apply. It is very reliable method to determining the drug susceptibility to pharmaceuticals.

MATERIALS AND METHODS

DRUG DESIGN

A binding interaction between a small molecule ligand and an enzyme protein results in activation or inhibition of the enzyme which results in agonism or antagonism.

GLIDE DOCKING

Glide is one of the docking programs which predicts the binding mode of ligand to a protein (target). It ranks the ligands via high-throughput virtual screening. Extra prediction mode (XP) was used to rank order the compounds based on the interaction with the receptors.

Protein preparation

Ligand preparation

Receptor grid generation

Ligand docking (screening)

MOLECULAR DOCKING BY ARGUS LAB SOFTWARE [37]

Docking of ligands is carried out by Argus lab docking software. Docking allows the medicinal chemist to virtually screen a set of compounds and predict the strongest binding capacity based on various scoring function. It explores ways in which two molecules such as ligand and receptor (protein) fit together and docks to each other well. The molecule binding to a receptor inhibits its function and thus acts as drug.

Argus lab 4.0 distributed freely for windows platforms by planaria software, is an introductory molecular modeling package with academics. Argus docking engine implementry in Argus lab approximates an exhaustive search method which is similar to DOCK and GLIDE. Flexible ligand docking is possible with Argus lab, where the ligand is described as torsion tree and grids are constructed that overlay the binding site. The accuracy of the Argus lab docking algorithm takes into account, the key features such as the nature of the binding site and the number of rotatable bonds to the ligand.

Molegro molecular viewer: Molegro molecular viewer is an application which helps in analyzing the energies and interaction of the binding site.

Q-site finder is an energy-based method for protein-ligand binding site prediction. During prediction we use the crystal structures of macromolecules (receptor) with small substrates (pdb ID). Identifying the location of binding sites on a protein is of fundamental importance for a range of applications including molecular docking. It uses the interaction energy between the protein and a simple vanderwaals probe to locate energetically favourable binding sites.

A .PREPARATION OF PROTEIN

STEP 1:

- Enter protein pdb ID (4ACF) in the protein data bank.
- Go to download files and select pdb as text file.
- Save the downloaded pdb text file to desktop.

STEP 2:

- Open Argus lab file → open → Import pdb file from the desktop.

- 3D structure of the protein will appear in the workspace of Argus lab.
- Left side of the screen shows molecular tree view.
- Open pdb → open 'Residues' → open 'Misc'.
- From 'Misc' delete the inhibitor and hetero residues, do not delete cofactor.
- Open water press shift, select all water molecules and delete.
- Add hydrogen atoms.
- Go to calculation on toolbar → energy by UFF method → start.
- Save the prepared protein as *.agl file format in the desktop.

B.IDENTIFICATION/ SELECTION OF ACTIVE SITE

STEP 1:

- Open Q-site finder through online.
- Upload/Import the pdb format of the protein.
- Find all the active site and make a list out of the common amino acid residues.

STEP 2:

- Open residues → open amino acids
- Press control and select the amino acids which were listed from the Q-site finder.

- Make sure that all the amino acid residues listed are selected.

- Right click on the mouse → make a group from the selected residues → give name → Binding site → OK.

C.PREPARATION OF LIGANDS

- Draw the structure from chem. Sketch and save as MDL mol format.

- Import the ligand into workspace of Argus lab.

- Clean geometry → clean hybridization.

- Select the ligand, right click on the mouse → make a group from the residues → give name→ ligand→ OK.

D.DOCKING PARAMETER

- Select calculation from the toolbar → Dock a ligand

- 'Argus Dock' as the Docking engine

- 'Dock' was selected as calculation type

- 'Flexible' for the ligand

- Ascore as the scoring function

- Calculation size

- Start docking

- Save the Docked protein Ligand complex as Brookhaven pdb files (*.pdb)

B.VISUALIZATION/INTERPRETATION OF DOCKING

Molegro molecular viewer helps in analyzing the energies and interaction of the binding. [38]

Lipinski's rule

Lipinski's rule of five is a rule of thumb to evaluate drug likeness, ie., or to determine if a chemical compound with a certain pharmacological or biological activity has the properties that would make it a likely orally active drug in humans. The rule was formulated by Christopher A. Lipinski in 1997, based on the observation that most medication drugs are relatively small and lipophilic molecules.

The rule describes molecular properties important for a drug's pharmacokinetics in the human body, including their absorption, distribution, metabolism and excretion (ADME). However, the rule does not predict if a compound is pharmacologically active.

The rule is important for the drug development where a pharmacologically active lead structure is optimized step-wise for increased activity and selectivity, as well as drug-like properties as described by Lipinski's rule. The modification of the molecular structure often leads to drugs with higher molecular weight, more rings, more rotatable bonds, and a higher lipophilicity.

Lipinski's rule says that, an orally active drug has no more than one violation of the following criteria:

Not more than 5 hydrogen bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms)

Not more than 10 hydrogen bond acceptors (nitrogen or oxygen atoms)

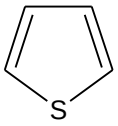
Molecular weight under 500 daltons

Partition coefficient of log P less than 5

HETEROCYCLIC CHEMISTRY

Heterocyclic structures always are a part in the field of research and development in organic chemistry. Millions of heterocyclic structures are found to exist having special properties and biological importance. Among various compounds, I have chosen thiophene a five membered ring containing sulfur moiety.

THIOPHENE NUCLEUS



The following synthetic reactions are involved for synthesizing thiophene containing compounds

Paal Knorr Synthesis
Hinsberg synthesis of thiophene
Gewald Reaction

SYNTHETIC METHODOLOGY

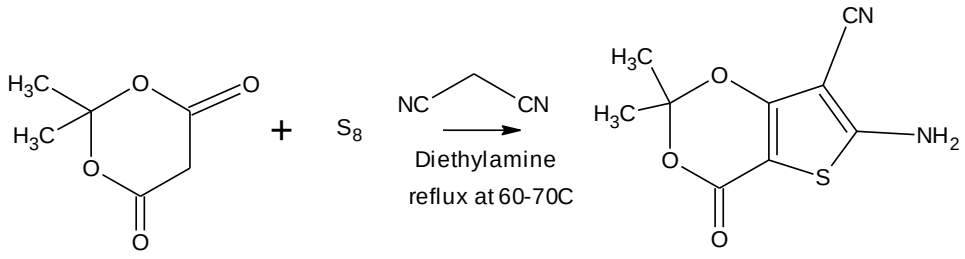
In this methodology, using Gewald synthetic process synthesizing novel compounds using different characterized alpha-methyl ketones, malononitrile and elemental sulfur, further reaction carried out by using ethanol as a solvent.

SYNTHETIC PROCEDURE

In this synthetic procedure, 1st step involved in alpha-methyl ketone like(meldrum's acid and cycloheptanone), malonitrile is dissolved in ethanol (used as a solvent), add diethylamine as a catalyst (half quantity of calculated amount) the reaction mixture stirred at room temperature for about 18hrs. Completion of the reaction is comfirmed by TLC and detected by UV chamber.

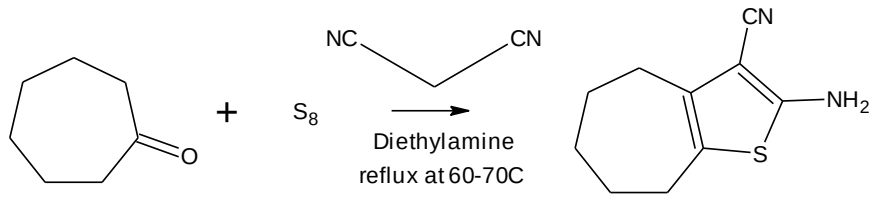
After completion of the reaction, in the reaction mixture remaining quantity of diethylamine and crushed elemental sulfur is added and reflux with stirring is carried out until sulfur completely dissolved and the temperature is maintained at 60 – 80^0C (over 5-6hrs). Then the mixture again stirred at room temperature for 12 hrs. The completion of the reaction was confirmed by TLC and the reaction mixture poured into crushed ice. Finally the product was obtained which was recrystallized by using various organic solvents like ethanol, ethyl acetate, etc.

SYNTHETIC REACTIONS



Meldrum's acid

6-amino-2,2-dimethyl-4-oxo-4*H*-thieno[3,2-*d*][1,3]dioxine-7-carbonitrile



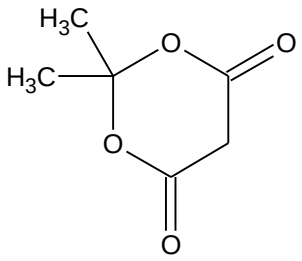
Cycloheptanone

2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carbonitrile

REACTANT PROFILE

MELDRUM'S ACID

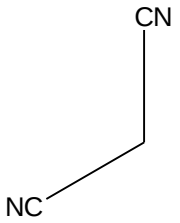
STRUCTURE



SYNONYM	Meldrum's acid
MOLECULAR FORMULA	C6H8O4
MOLECULAR WEIGHT	144.15
SOLUBILITY	Soluble in Ethanol, ethyl acetate, methanol
DESCRIPTION	Yellowish white salt (hygroscopic)
MELTING POINT	192-194^{0}C

MALONITRILE

STRUCTURE



SYNONYM	Malonitrile
MOLECULAR FORMULA	C3H2N2
SOLUBILITY	Soluble in ethanol and sparingly soluble in ethyl acetate
DESCRIPTION	Liquid in nature (reddish brown)

DIETHYLAMINE

SYNONYM	Diethylamine
MOLECULAR FORMULA	73.14
SOLUBILITY	Soluble in ethanol
DESCRIPTION	Colorless liquid

SYNTHETIC DERIVATIVES

The derivatives of the compounds were synthesized by following procedure

6-AMINO-2,2-DIMETHYL-4-OXO-4H-THIENO(3,2-d)(1,3)DIOXINE-7-CARBONITRILE

2-AMINO-5,6,7,8-TETRAHYDRO-4H-CYCLOHEPTA(b)THIOPHENE-3-CARBONITRILE

The above synthesized compounds (intermediate) are again reacted with following acids like glacial acetic acid, formic acid and carbondisulphide.

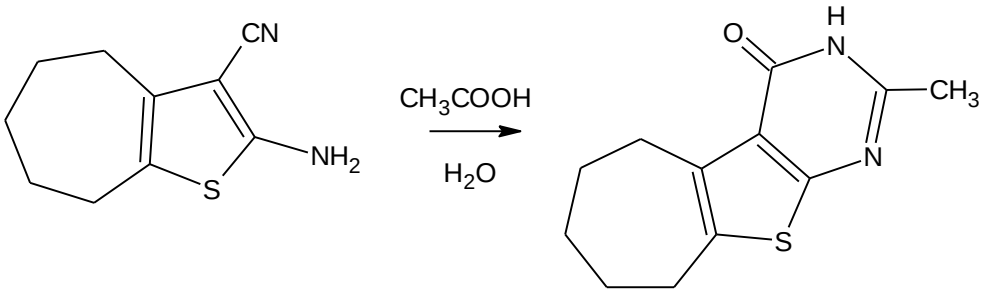
In the compound A and B, add 20ml of formic acid and 3 drops of conc.H_2 SO_4, temperature is maintained at 80^0C for 12hrs, pyrimidine analogue is formed.

In the compound A and B,10ml of carbondisulphide and ethanolic potassium hydroxide as a solvent and the reaction is carried out reflux with stirring for 12hrs, temperature maintained at 80^0C

In the compound A and B, add 30ml of glacial acetic acid (used as a solvent), and the temperature is maintained at 80^0C for 12hrs 2 methyl pyrimidine analogue is formed.

REACTION:

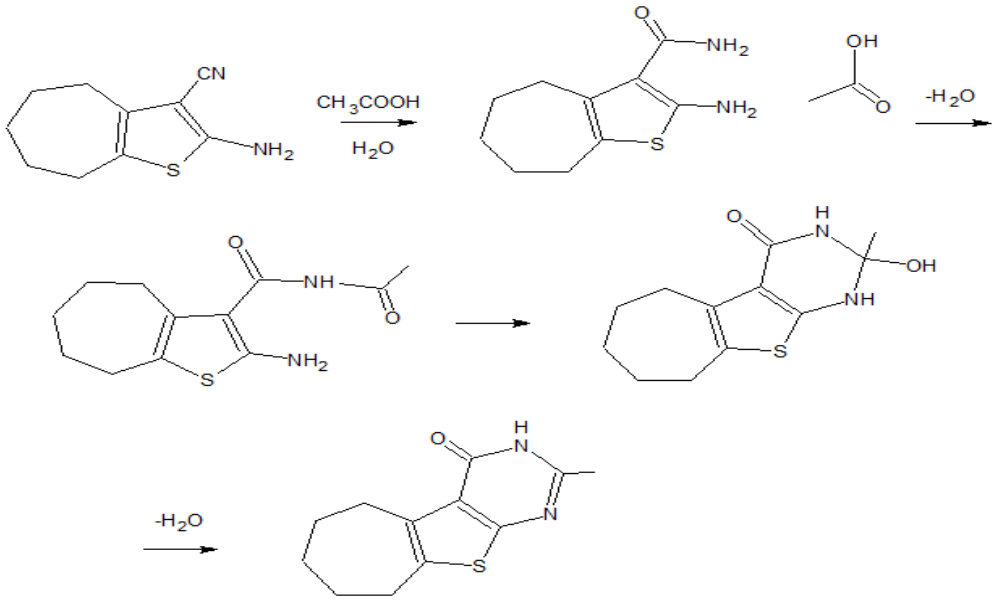
COMPOUND NAME: CMG



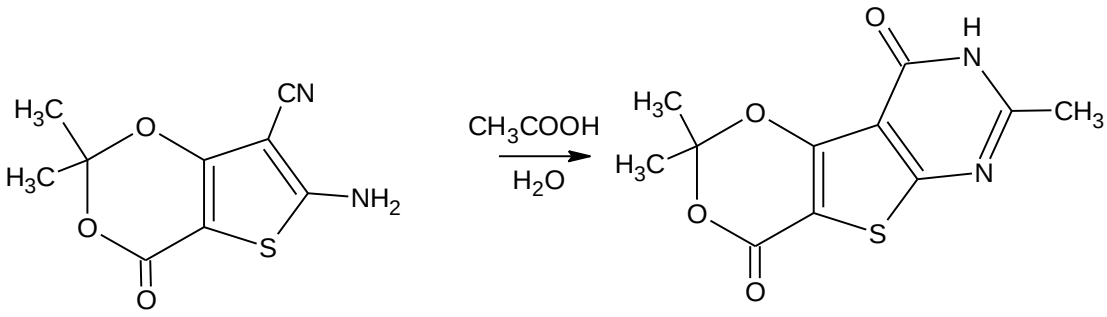
2-amino-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carbonitrile

2-methyl-4a,5,6,7,8,9-hexahydro-4H-cyclohepta[4,5]thieno[2,3-d]pyrimidi

MECHANISM



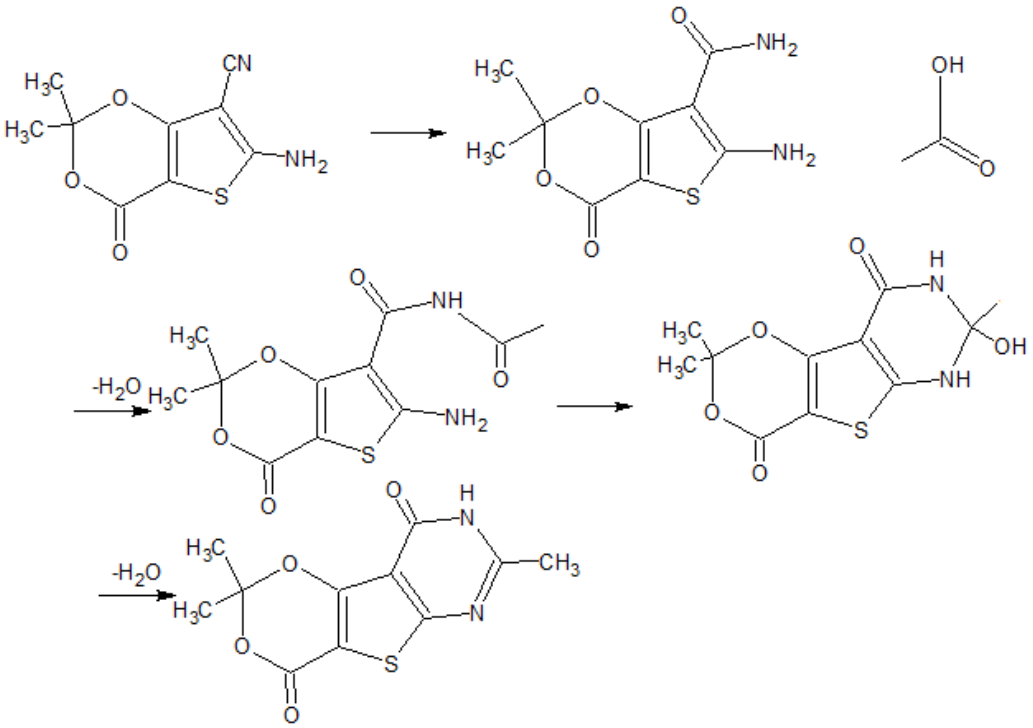
COMPOUND NAME: MMG



6-amino-2,2-dimethyl-4-oxo-4H-thieno
[3,2-d][1,3]dioxine-7-carbonitrile

2,2,7-trimethyl-9,9a-dihydro-4H-[1,3]
dioxino[4',5':4,5]thieno[2,3-d]pyrimidin-4-one

MECHANISM



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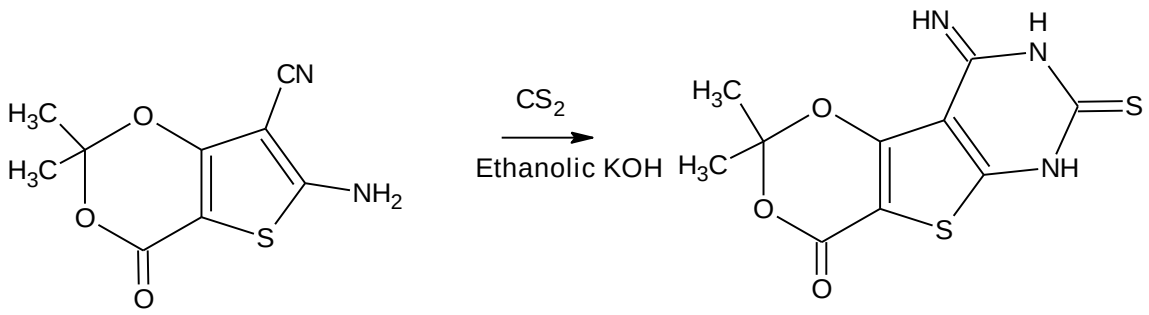
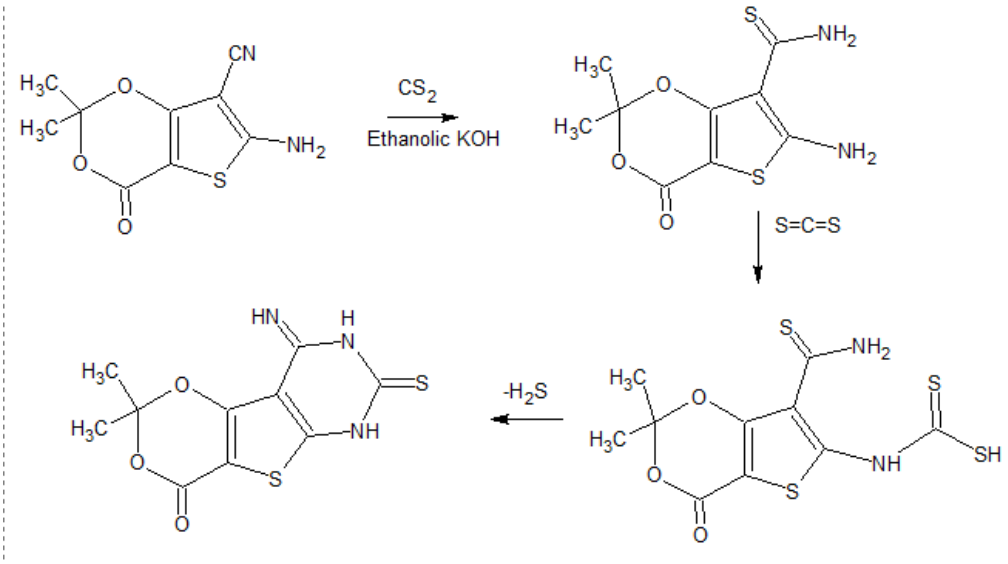


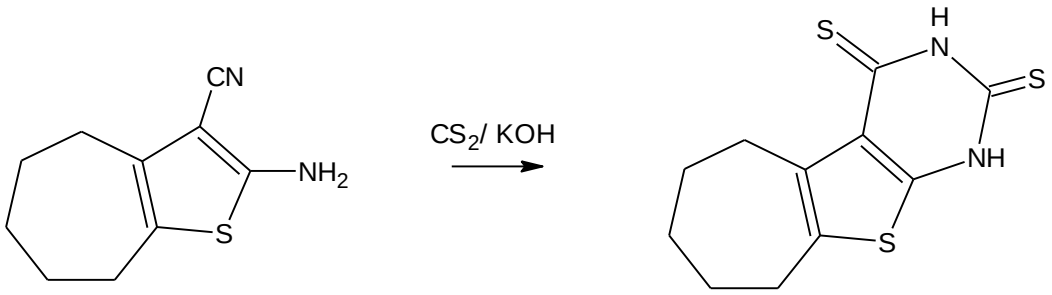
6-amino-2,2-dimethyl-4-oxo-4H-thieno [3,2-d][1,3]dioxine-7-carbonitrile

9-imino-2,2-dimethyl-7-thioxo-6,9-dihydro-4H,7H-[1,3]dioxino[4',5':4,5]thieno[2,3-d][1,3]thiazin-4-one

MECHANISM



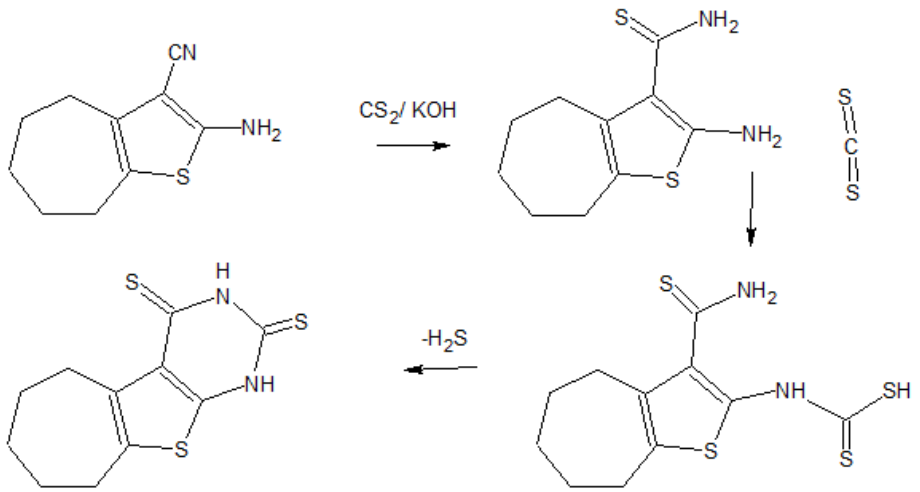
COMPOUND NAME: CC



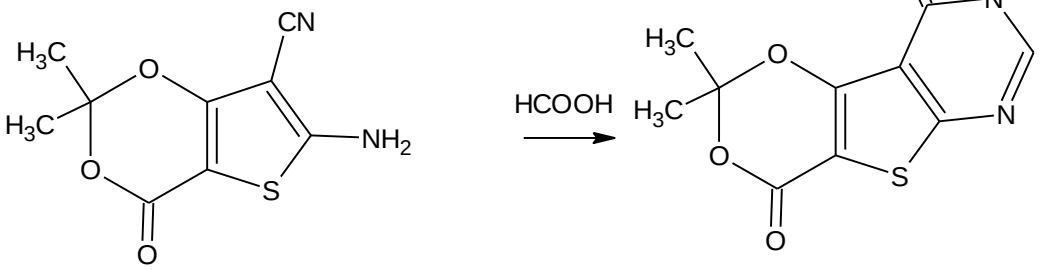
2-amino-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carbonitrile

4-imino-1,5,6,7,8,9-hexahydro-2H,4H-cyclohepta[4,5]thieno[2,3-d][1,3]thiazine-2-thione

MECHANISM



COMPOUND NAME: MMF



6-amino-2,2-dimethyl-4-oxo-4H-thieno [3,2-d][1,3]dioxine-7-carbonitrile

2,2-dimethyl-9,9a-dihydro-4H-[1,3] dioxino[4',5':4,5]thieno[2,3-d]pyrimidin-4-one

METHODS OF IDENTIFICATION

The synthesized compounds were identified by using following methods.

Melting point

The melting point of the compounds are determined by the capillary tube method. The synthesized compounds were start losing their crystallinity at a particular temperature.

Thin layer chromatography

Pre-coated TLC plates with silicagel GF 250 are used. Samples of reactants and products are prepared with suitable solvents.

The characterization was carried out using sophisticated methods like Infra-red spectroscopy, Nuclear magnetic resonance spectroscopy and Mass spectroscopy.

INFRA RED ABSORPTION SPECTROSCOPY

IR (region 2.5-15) is a powerful tool for identifying pure organic and inorganic compounds because. With the exception of a few homo nuclear molecules such as O2, N2, Cl2 all the molecular species absorb infrared radiation. With the exception of chiral molecules in the crystalline state, each molecular species has a unique infrared absorption spectrum.

NMR SPECTRA

Nuclear magnetic resonance involves the interaction between oscillating magnetic field of electromagnetic radiation and the magnetic energy of the hydrogen nucleus or some other type of nuclei when these are placed in an external static magnetic field.

NMR spectroscopy in which radiofrequency waves induces transitions between magnetic energy levels of nuclei of a molecule. NMR enables us to study the number of equivalent protons and their electronic environment. It reveals the different chemical environment in which the proton is present and helps us to ascertain the structure of molecule

The number of signals in an NMR spectrum denotes the number of the set of equivalent protons in a molecule. The position of the signals in the spectrum helps us to know the nature of protons such as aromatic, aliphatic, acetylenic, vinyl, adjacent to some electron attracting or electron releasing group etc. the splitting of the signal is due to the different environment of the absorbing proton with respect to the adjacent protons and not with respect to electrons.

MASS SPECTROSCOPY

Mass spectroscopy is an analytical techniques used to establish the molecular structure and the molecular weight of the analyte under investigation. In this technique, the compound under investigation is bombarded with a beam of electrons producing ionic fragments of the original species. The relative abundance of the fragment ion formed depends on the stability of the ion and of the lost radical. The resulting charged particles are

then separated according to their masses. Mass spectrum is a record of information regarding various masses produced and their relative abundances.

BIOLOGICAL SCREENING FOR ANTI-TB ACTIVITY

This screening is for the compounds that have the potential to be developed in to new drugs against tuberculosis because the compounds inhibit the enzymes required for the formation of cell wall of the tuberculosis bacterium. New drugs are needed in current date, because the rates of cure with present drugs are very slow, and prevalence of mycobacterium tuberculosis resistance to the present drugs is increasing.

ANTITUBERCULAR ACTIVITY

The following methods have been used for invitro studies for the evaluation of anti-tubercular activity.

- Resazurin Micro plate Assay (REMA)
- Nitrate Reductase Assay (NRA)
- Micro plate Alamar Blue Assay (MABA)
- 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)
- Middle Brook 7H11 Agar dilution Assay
- Broth Micro dilution Method
- BACTEC system
- Luciferase Reporter Phage Assay

The synthesized compounds may be evaluated for anti-tubercular activity using any of the above methods. In this techniques, Microplate Alamar Blue Assay is used to evaluate the anti-tubercular activity.

MICROPLATE ALAMAR BLUE ASSAY

Alamar blue dye is used as an indicator for the determination of viable cells.

The oxidized form, Resazurine (also called as diazo-resorcinol, azoresorcin, resazoin and resazurine) is non- toxic, non-fluorescent and blue in colour which becomes pink and fluorescent upon reduction to resorufin by viable cells.

Growth is measured qualitatively by a visual colour change and the amount of fluorescence produced is proportional to the number of the living cells which is determined by colorimetric and fluorimetric methods.

Advantages

- ❖ Easy to use
- ❖ High sensitivity
- ❖ Does not require cell lysis
- ❖ Works well with different types of cells
- ❖ Safe and economical
- ❖ Inexpensive
- ❖ Results are linear and quantitative

ASSAY PROCEDURE FOR ESTIMATING ANTI-TB ACTIVITY USING Alamar Blue DYE

- The anti-mycobacterial activity of compounds is assessed against M. tuberculosis using Alamar Blue micro plate assay (MABA).
- This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method.
- Briefly, 200 micro litter of sterile de-ionized water is added to all outer perimeter wells of sterile 96 wells plate to minimize evaporation of medium in the test wells during incubation.

- The 96 wells plate receive 100 micro litter of the middle brook 7H9 broth and serial dilution of compounds are made directly on plate.
- The final drug concentrations tested are 100 to 0.2 micro gram/ml.
- Plates are covered and sealed with paraffin and incubated at 37C for five days.
- After this time, 25 micro litters of freshly prepared 1:1 mixture of Alamar blue reagent and 10% tween 80 is added to the plate and incubated for 24 hrs.
- A blue colour in the well is interpreted as no bacterial growth, and pink colour was scored as growth.
- The MIC is defined as lowest drug concentration which prevents the colour change from blue to pink.

IN SILICO TOXICITY PREDICTION

In silico toxicity prediction is done using OSIRIS Property Explorer. It is free software available for access in the Organic Chemistry Portal. Using this prediction tool, mutagenicity, tumerogenicity, skin irritation and reproductive effects can be calculated. The prediction properties relies on a precompiled set of structure fragment that gives rises to toxicity alerts in case they are encountered in the structure currently drawn. These fragment lists is created by rigorously shredding all compounds in the data base known to be active in a certain toxicity class. During the shredding any molecule is first cut at every rotatable bonds leading to a set of core fragments.

The designed and docked molecules are screened in silico using MOLINSPIRATION

Cheminformatics software to evaluate drug likeness. This tool is quick and easy to use. It is a software available online for calculation of important molecular properties 9logP, polar surface area, number of hydrogen bond donors and acceptors and others, as well as prediction of bioavailability score for the most important drug targets(GPCR ligands, Kinase inhibitors, ion channel modulators, nuclear receptors)

RESULTS AND DISSCUSSION

Nearly 200 molecules were sketched using chem sketch, and the molecules which is novel to be docked against the enzyme using Argus Lab 4.0.1 software.

The following molecules were docked against different targets and the molecules with best docking score and good interaction were selected and synthesis.

Molecules docked against different targets;

1. Glutamine Synthetase I

2. Methoxy Mycolic acid Synthase II

3. Cyclopropane Mycolic Acid Synthase II

4. L,D-Transpeptidase

5. Decaprenyl Phosphoryl-b-d-Ribose2'-EpimeraseI (DprEI)

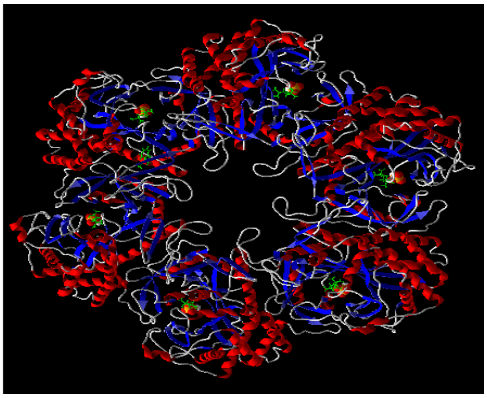
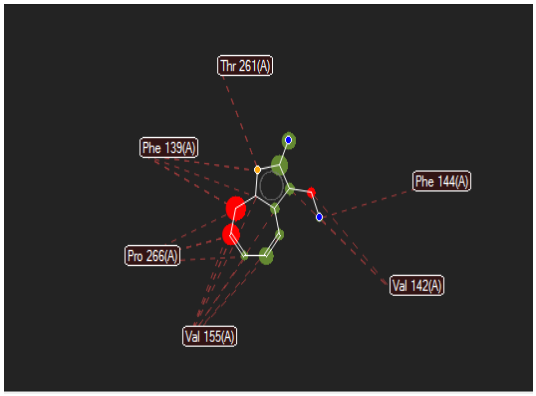
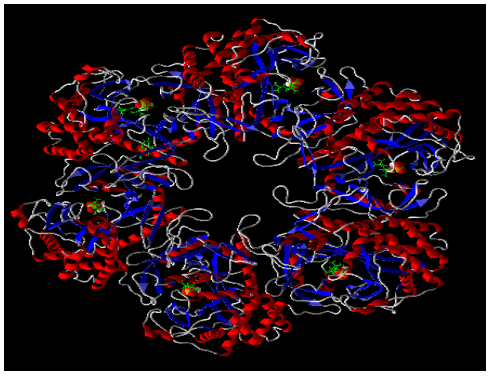
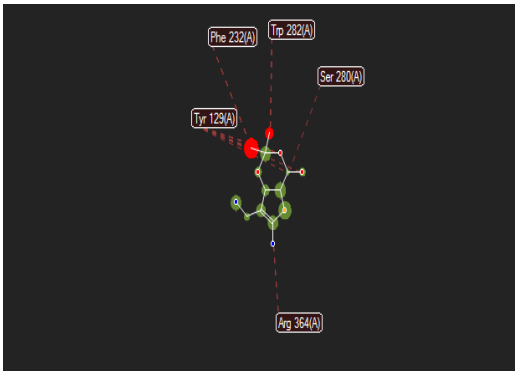
TABLE NO 1: DOCKING SCORE OF THE SYNTHESIZED COMPOUNDS

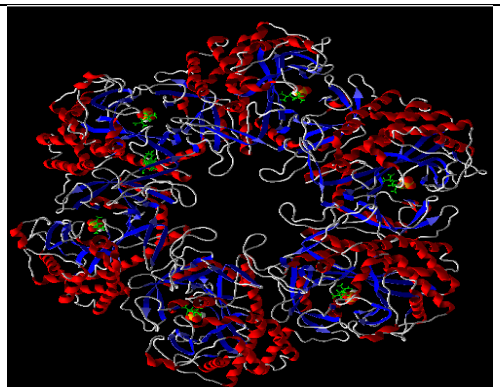
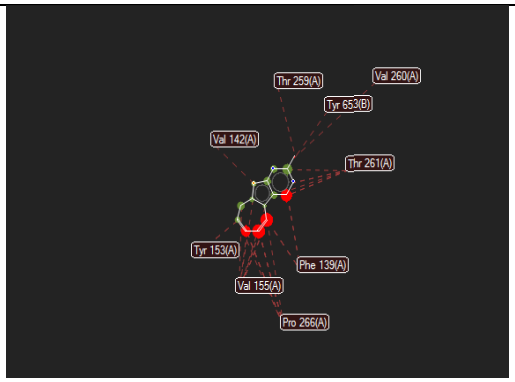
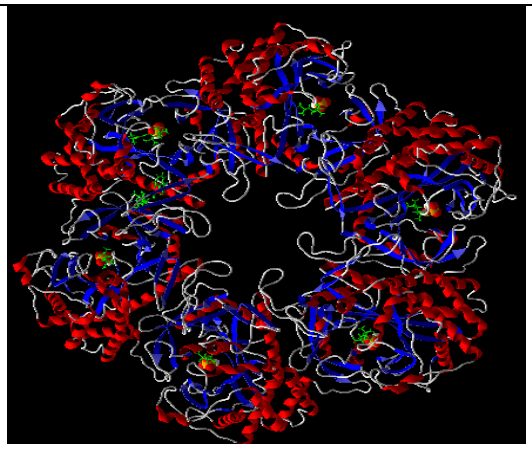
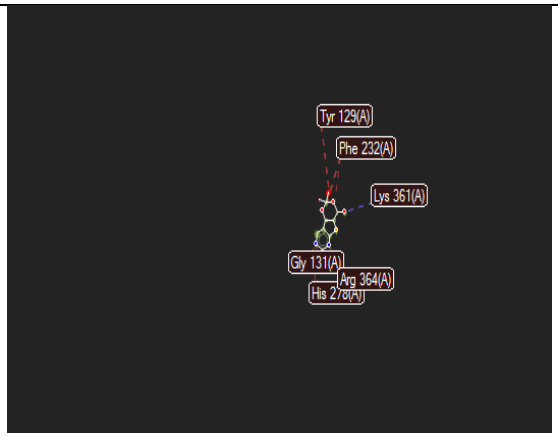
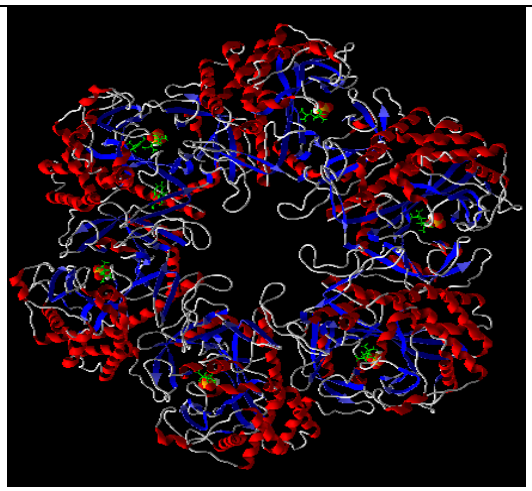
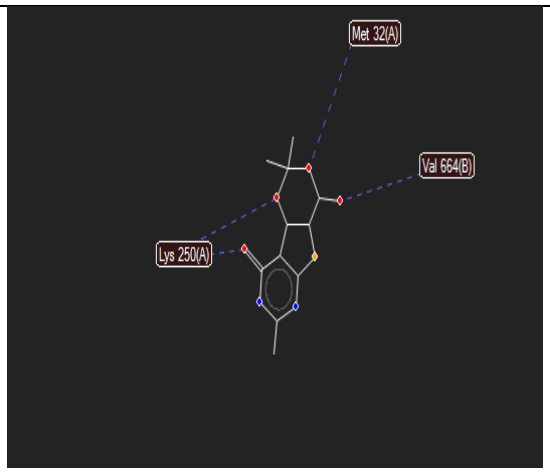
NAME OF THE ENZYMES	DOCKING SCORES(Kcals/mol)				
	CMG	MMG	MMF	MC	CC
GLUTAMINE SYNTHETASE I	-7.4572	-7.4632	-7.8345	-7.5543	-9.3876
METHOXY MYCOLIC ACID SYNTHASE I	-8.8865	-9.6543	-7.9854	-8.7651	-9.7641
CYCLOPROPANE MYCOLIC ACID SYNTHASE	-7.3218	-7.3490	-8.6721	-7.7423	-9.9732
L,D-TRANSPEPTIDASE	-6.7842	-7.2143	-6.9543	-6.8974	-7.7542
DprEI	-7.8352	-6.9874	-7.5632	-7.8974	-7.5417

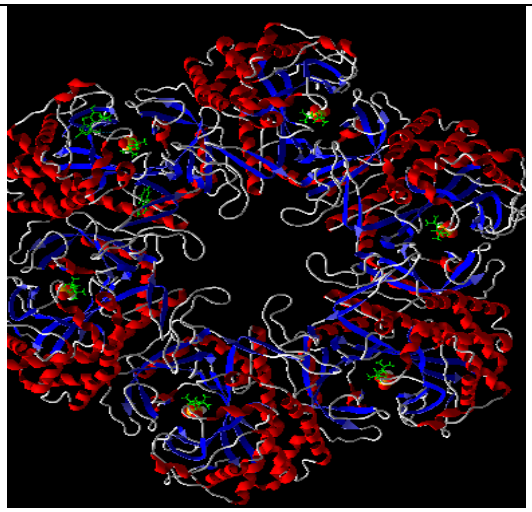
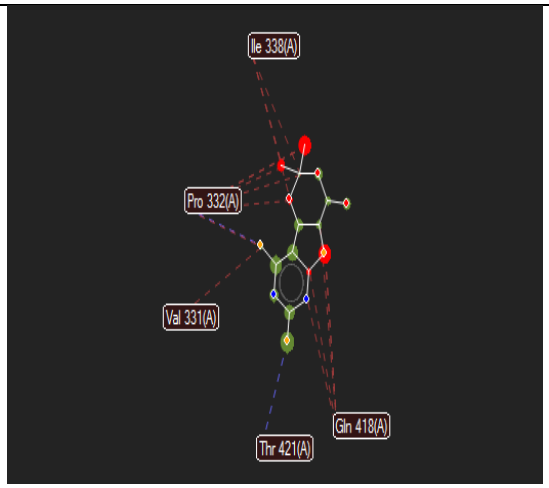
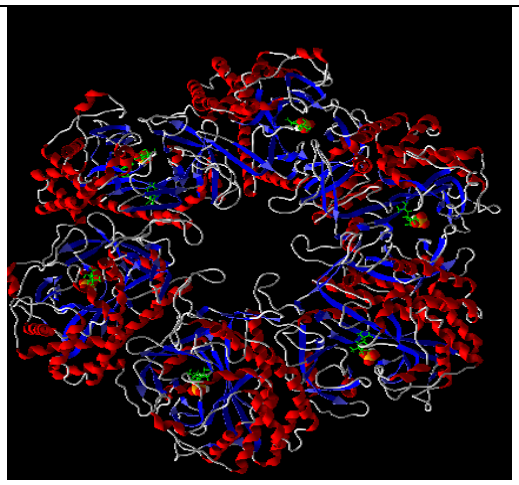
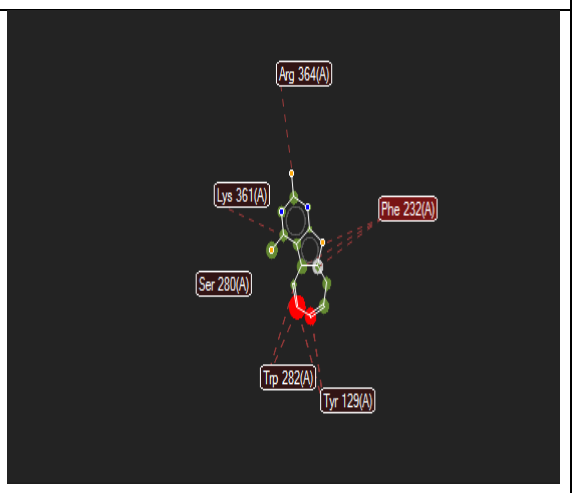
INTERACTION OF THE DOCKED MOLECULES WITH THE ENZYME

GLUTAMINE SYNTHETASE

TABLE NO 2: DOCKING VIEW AND THEIR INTERACTION

SAMPLE CODE	DOCKING VIEW	INTERACTION WITH AMINOACIDS
CM		
MM		
CMG		

		 Thr 259(A) Val 260(A) Tyr 653(B) Val 142(A) Thr 261(A) Tyr 153(A) Phe 139(A) Val 155(A) Pro 266(A)
MMG		 Tyr 129(A) Phe 232(A) Lys 361(A) Gly 131(A) Arg 364(A)
MMF		 Met 32(A) Val 664(B) Lys 250(A)

MC		
CC		

RESULTS OF SCHEMES

Physicochemical properties of 2-methyl-4a,5,6,7,8,9-hexahydro-4cyclohepta[4,5]thieno[2,3-*d*]pyrimidine Compound name: CMG

Molecular Formula = $C_{12}H_{16}N_2S$

Formula Weight = 220.335

Composition = C(65.41%) H(7.32%) N(12.71%) S(14.55%)

Molar Refractivity = 63.93 ± 0.5 cm^3

Molar Volume = 163.2 ± 7.0 cm^3

Parachor = 434.7 ± 8.0 cm^3

Index of Refraction = 1.711 ± 0.05

Surface Tension = 50.2 ± 7.0 dyne/cm

Density = 1.34 ± 0.1 g/cm^3

Dielectric Constant = Not available

Polarizability = 25.34 ± 0.5 $10^{-24} cm^3$

Monoisotopic Mass = 220.103419 Da

Nominal Mass = 220 Da

Average Mass = 220.337893 Da

Physicochemical properties of 2,2,7-trimethyl-9,9a-dihydro[1,3]dioxino[4',5':4,5]thieno[2,3-*d*]pyrimidin-4-one Compound name: MMG

Molecular Formula = $C_{11} H_{12} N_2 O_3 S$

Formula Weight = 252.291

Composition = C(52.37%) H(4.79%) N(11.10%) O(19.02%) S(12.71%)

Molar Refractivity = 63.11 ± 0.5 cm^3

Molar Volume = 161.1 ± 7.0 cm^3

Parachor = 440.0 ± 8.0 cm^3

Index of Refraction = 1.712 ± 0.05

Surface Tension = 55.6 ± 7.0 dyne/cm

Density = 1.56 ± 0.1 g/cm^3

Dielectric Constant = Not available

Polarizability = 25.02 ± 0.5 $10^{-24} cm^3$

Monoisotopic Mass = 252.056864 Da

Nominal Mass = 252 Da

Average Mass = 252.292867 Da

Physicochemical properties of 2,2-dimethyl-9,9a-dihydro-4[1,3]dioxino[4',5':4,5]thieno[2,3-*d*]pyrimidin-4-one Compound name: MMF

Molecular Formula = $C_{10} H_{10} N_2 O_3 S$

Formula Weight = 238.264

Composition = C(50.41%) H(4.23%) N(11.76%) O(20.14%) S(13.46%)

Molar Refractivity = 58.69 ± 0.5 cm^3

Molar Volume = 145.9 ± 7.0 cm^3

Parachor = 408.9 ± 8.0 cm^3

Index of Refraction = 1.737 ± 0.05

Surface Tension = 61.7 ± 7.0 dyne/cm

Density = 1.63 ± 0.1 g/cm^3

Dielectric Constant = Not available

Polarizability = 23.26 ± 0.5 $10^{-24} cm^3$

Monoisotopic Mass = 238.041214 Da

Nominal Mass = 238 Da

Average Mass = 238.265878 Da

Physicochemical properties of 9-imino-2,2-dimethyl-7-thioxo-6,9-dihydro-4*H*,7*H*-[1,3]dioxino[4',5':4,5]thieno[2,3-*d*][1,3]thiazin-4-one Compound name: MC

Molecular Formula = $C_{10} H_8 N_2 O_3 S_3$

Formula Weight = 300.380

Composition = C(39.98%) H(2.68%) N(9.33%) O(15.98%) S(32.03%)

Molar Refractivity = 72.68 ± 0.5 cm^3

Molar Volume = 161.2 ± 7.0 cm^3

Parachor = 474.6 ± 8.0 cm^3

Index of Refraction = 1.861 ± 0.05

Surface Tension = 75.1 ± 7.0 dyne/cm

Density = 1.86 ± 0.1 g/cm^3

Dielectric Constant = Not available

Polarizability = 28.81 ± 0.5 $10^{-24} cm^3$

Monoisotopic Mass = 299.969706 Da

Nominal Mass = 300 Da

Average Mass = 300.378703 Da

Physicochemical properties of 4-imino-1,5,6,7,8,9-hexahydro-2*H*,cyclohepta[4,5]thieno[2,3-*d*][1,3]thiazine-2-thione Compound name: CC

Molecular Formula = $C_{11} H_{12} N_2 S_3$

Formula Weight = 268.424

Composition = C(49.22%) H(4.51%) N(10.44%) S(35.84%)

Molar Refractivity = 73.50 ± 0.5 cm^3

Molar Volume = 163.3 ± 7.0 cm^3

Parachor = 469.3 ± 8.0 cm^3

Index of Refraction = 1.858 ± 0.05

Surface Tension = 68.1 ± 7.0 dyne/cm

Density = 1.64 ± 0.1 g/cm^3

Dielectric Constant = Not available

Polarizability = 29.14 ± 0.5 $10^{-24}cm^3$

Monoisotopic Mass = 268.016261 Da

Nominal Mass = 268 Da

Average Mass = 268.423729 Da

IR SPECTROSCOPY

The samples were prepared by the KBr pellet techniques and the samples are analysed by FT-IR technique.

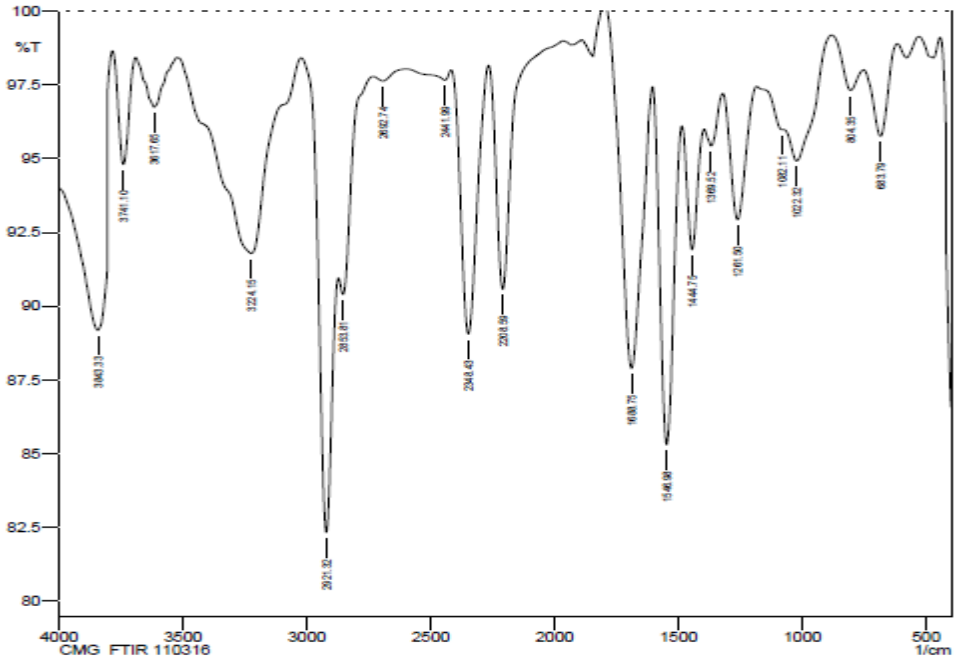
The spectra were examined for the absence of the absence of the functional groups of the parent compounds and examined for the presence of vibrational absorption band for the new functional groups.

The synthetic reaction involves, the reaction between 2-amino thiophene derivative with glacial acetic acid, formic acid and carbondisulphide to yield pyrimidine analogues

TABLE NO 3: IR ABSORPTION BAND

ABSORPTION BAND	CMG	MMG	MC	TM
CN Stretching	✓	✓	✓	✓
NH Stretching	✓	✓	✓	✓
C=N Stretching	✓	✓	×	✓
C=O Stretching	✓	✓	✓	✓

SAMPLE CODE: CMG



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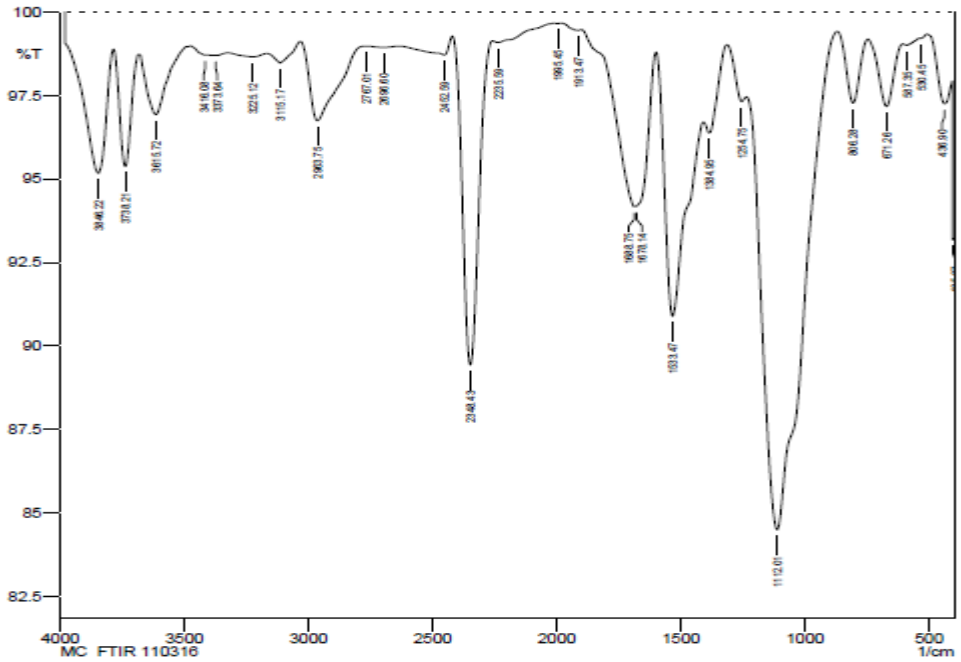
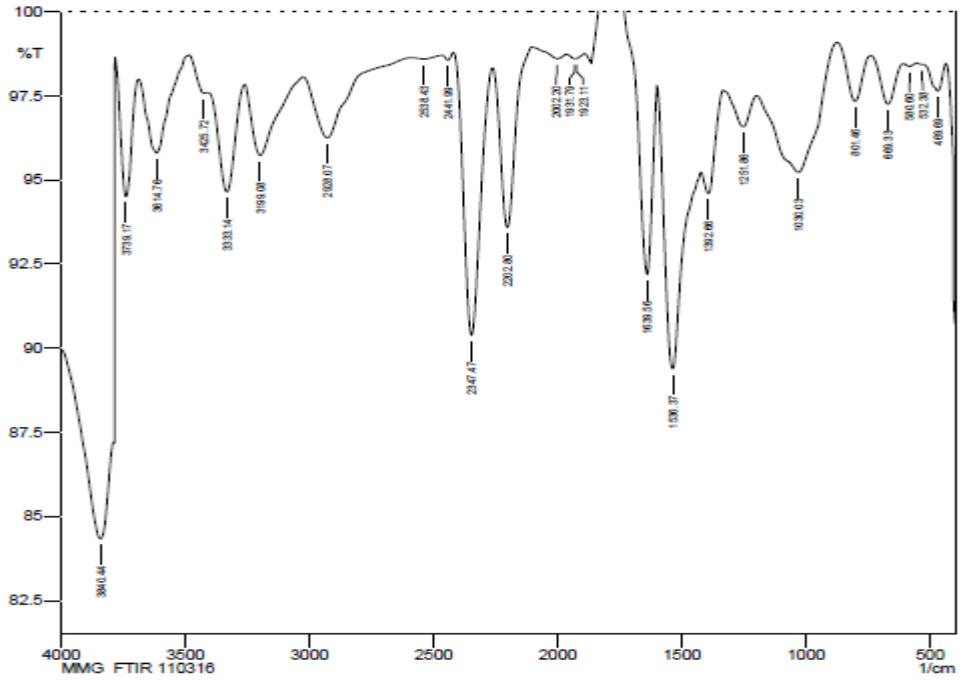
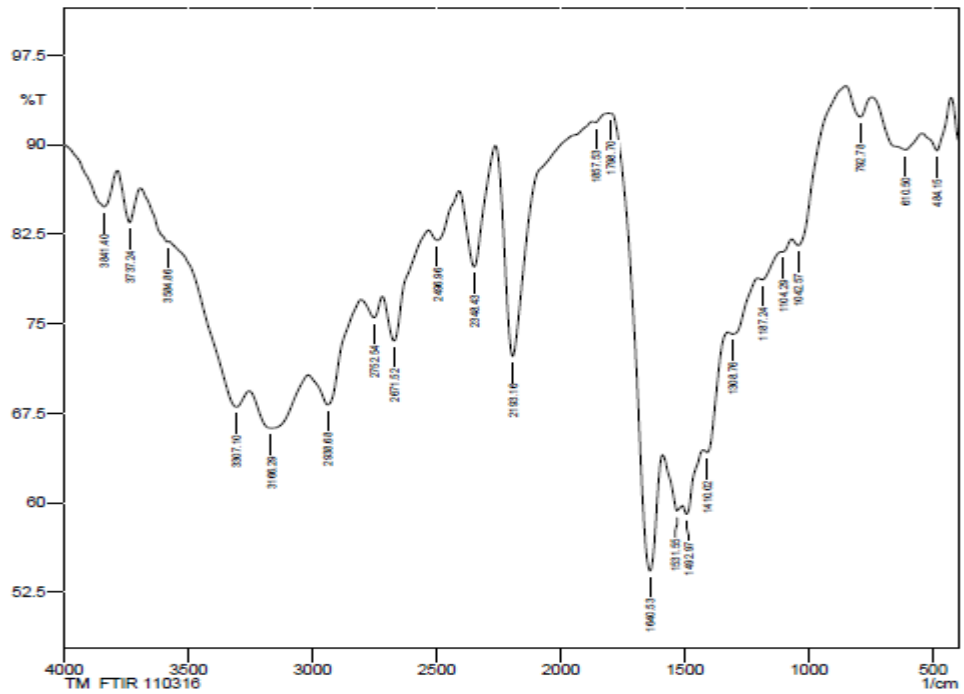


SAMPLE CODE MMG

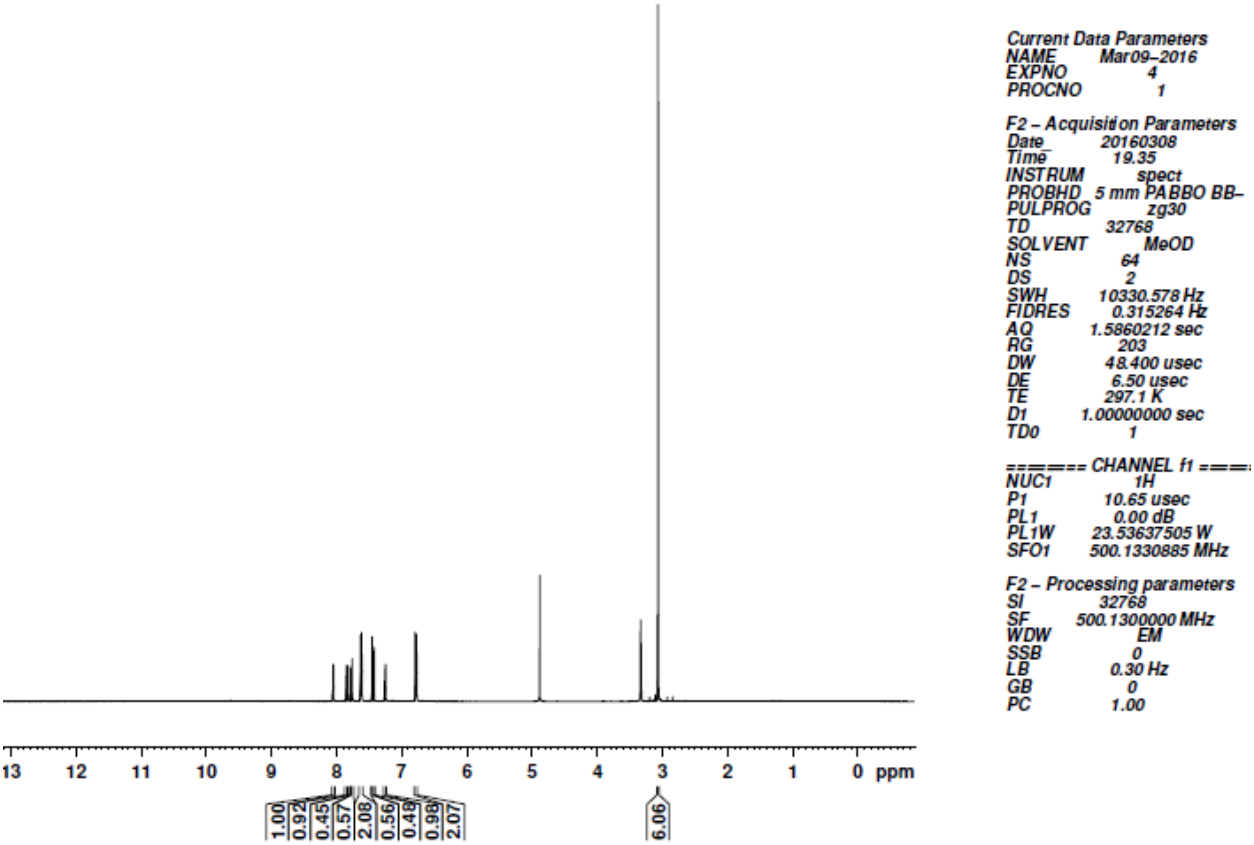


SAMPLE CODE TM



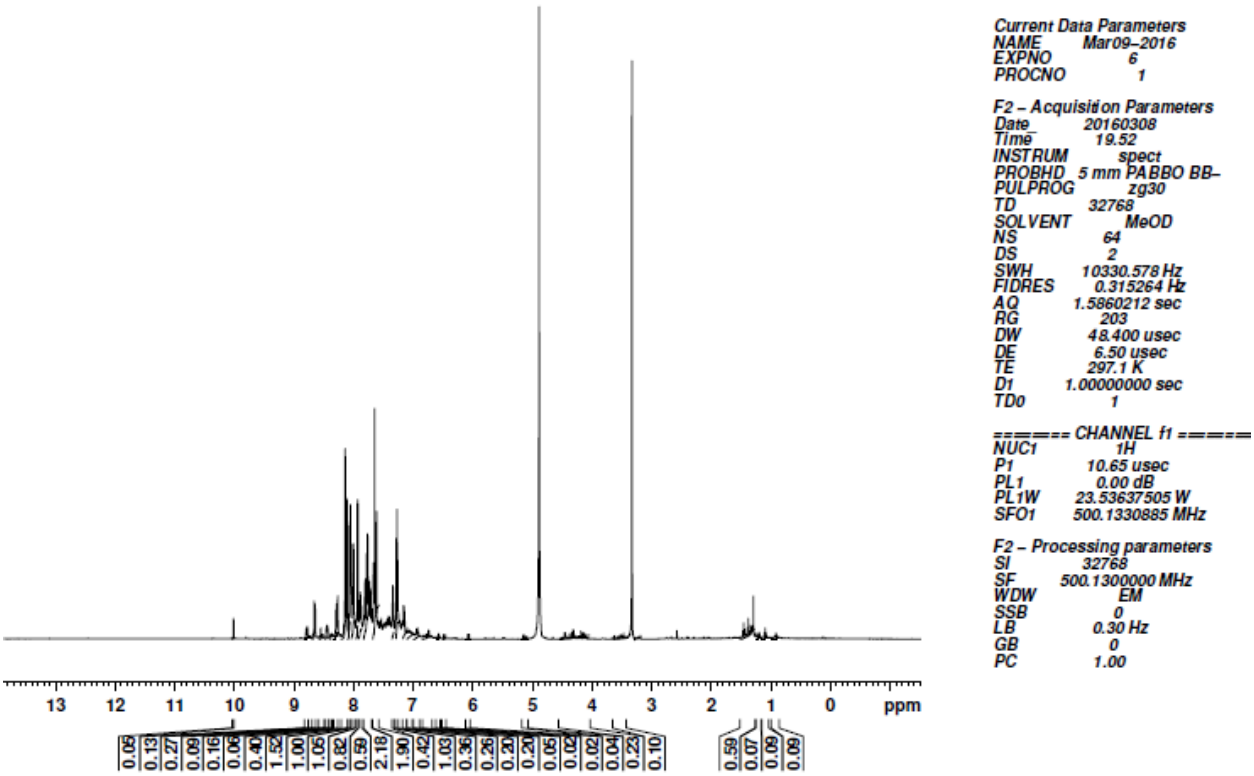
NMR SPECTROSCOPY

SAMPLE CODE: MC



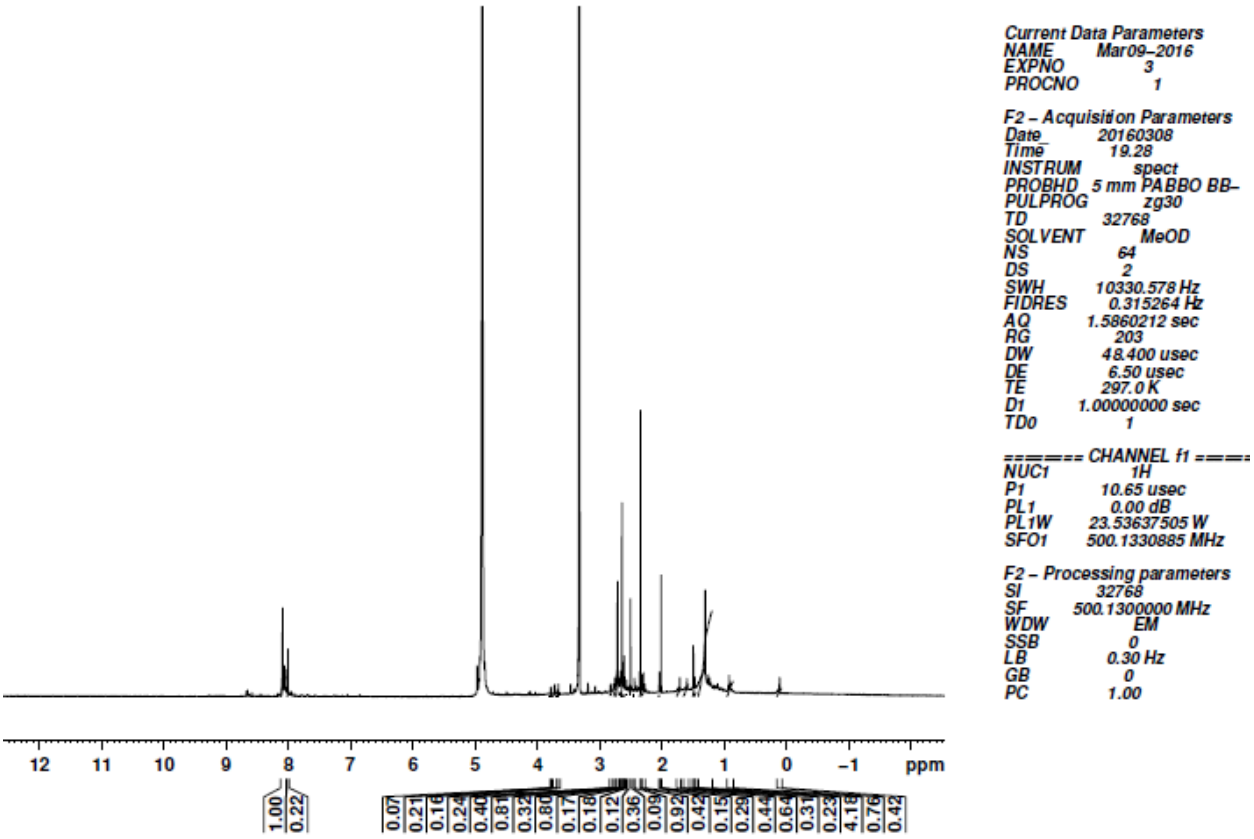
S.NO	Δ VALUE	TYPES OF PEAK	NUMBER OF PROTONS
1.	3.01	singlet	6
2.	6.78	doublet	2
3.	7.24-7.25	multiplet	1
4.	7.45	singlet	1
5.	7.62	doublet	2
6.	7.75	singlet	1
7.	7.83-7.85	multiplet	1
8.	8.04-8.05	Multiplet	1

SAMPLE CODE: CM



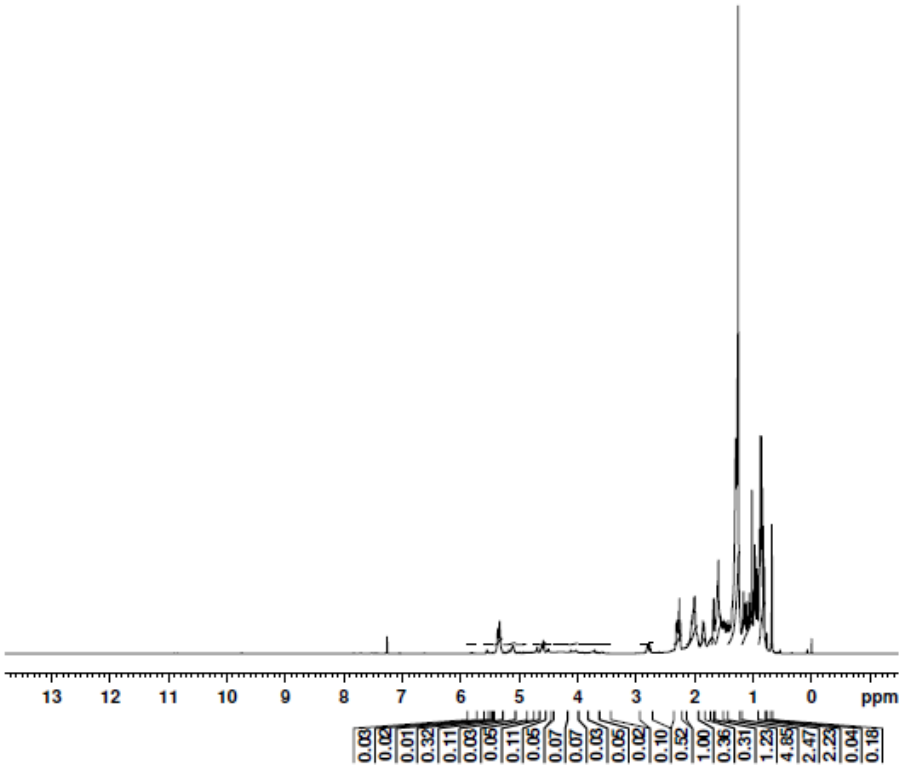
S.NO	Δ VALUE	TYPES OF PEAK	NUMBER OF PROTONS
1.	7.27-7.30	multiplet	1
2.	7.65	singlet	2
3.	7.71-7.78	multiplet	2
4.	7.85-7.87	multiplet	1
5.	7.93-7.96	multiplet	1
6.	8.00-8.07	multiplet	1
7.	8.11	singlet	1
8.	8.13-8.15	triplet	1

SAMPLE CODE: MMF



S.NO	Δ VALUE	TYPES OF PEAK	NUMBER OF PROTONS
1.	0.92	Triplet	1
2.	1.12-1.43	Multiplet	4
3.	1.61	Doublet	1
4.	2.34	Singlet	1
5.	2.66	Singlet	1
6.	2.70-2.72	Multiplet	1
7.	8.03-8.09	Multiplet	1

SAMPLE CODE: CMG



Current Data Parameters
NAME Feb12–2016
EXPNO 28
PROCNO 1

F2 – Acquisition Parameters
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Time 1.08
INSTRUM spect
PROBHD 5 mm PABBO BB–
PULPROG zg30
TD 32768
SOLVENT CDCl3
NS 32
DS 2
SWH 10330.578 Hz
FIDRES 0.315264 Hz
AQ 1.5860212 sec
RG 20.2
DW 48.400 usec
DE 6.50 usec
TE 297.5 K
D1 1.00000000 sec
TD0 1

======== CHANNEL f1 ======
NUC1 1H
P1 10.65 usec
PL1 0.00 dB
PL1W 23.53637505 W
SFO1 500.1330885 MHz

F2 – Processing parameters
SI 32768
SF 500.1300101 MHz
WDW EM
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LB 0.30 Hz
GB 0
PC 1.00

S.NO	Δ VALUE	TYPES OF PEAK	NUMBER OF PROTONS
1.	0.82-1.05	Singlet	2
2.	1.25	Doublet	2
3.	1.35-1.38	Multiplet	5
4.	1.41	Singlet	1
5.	2.01	Singlet	1
6.	2.23	Doublet	1

GC-MS SPECTROSCOPY

The molecular weight of synthesized compounds are confirmed by GC-MS Analysis.

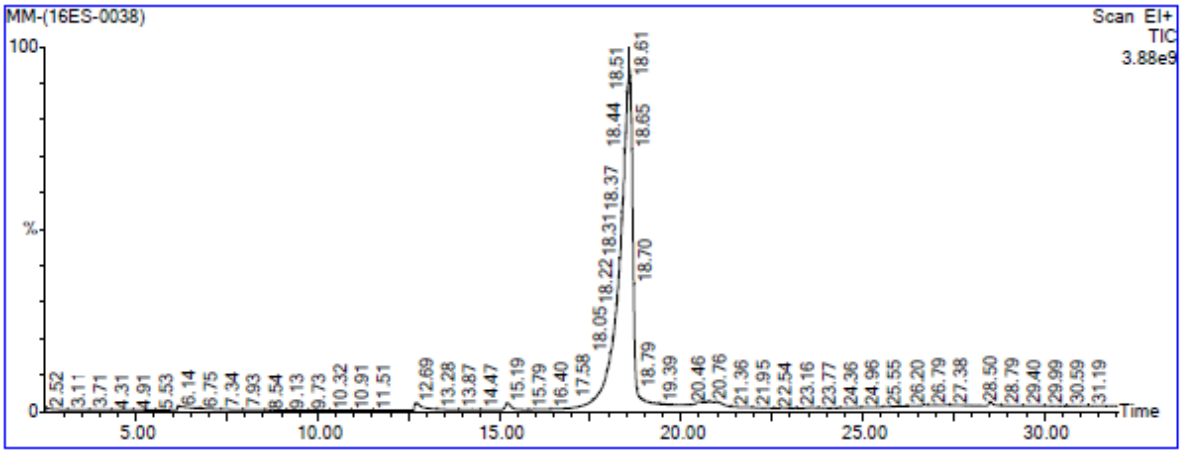
TABLE NO 4: GC-MS ANALYSIS

SAMPLE CODE	CALCULATED MASS	ACTUAL MASS
CM	192.28g/mol	192.13g/mol
MM	224.23g/mol	223.83g/mol
CMG	220.33g/mol	220.32g/mol
MMG	266.27g/mol	266.26g/mol
MMF	238.26g/mol	238.62g/mol
MC	300.38g/mol	300.37g/mol
CC	270.44g/mol	270.44g/mol

SAMPLE CODE: MM

GC/MS Method: GC: METHOD-1.mth MS: METHOD-1.EXP
Sample ID: MM-(16ES-0038)

Page 1 of 1
Vial Number: 75



#	RT	Scan	Height	Area	Area %	Norm %
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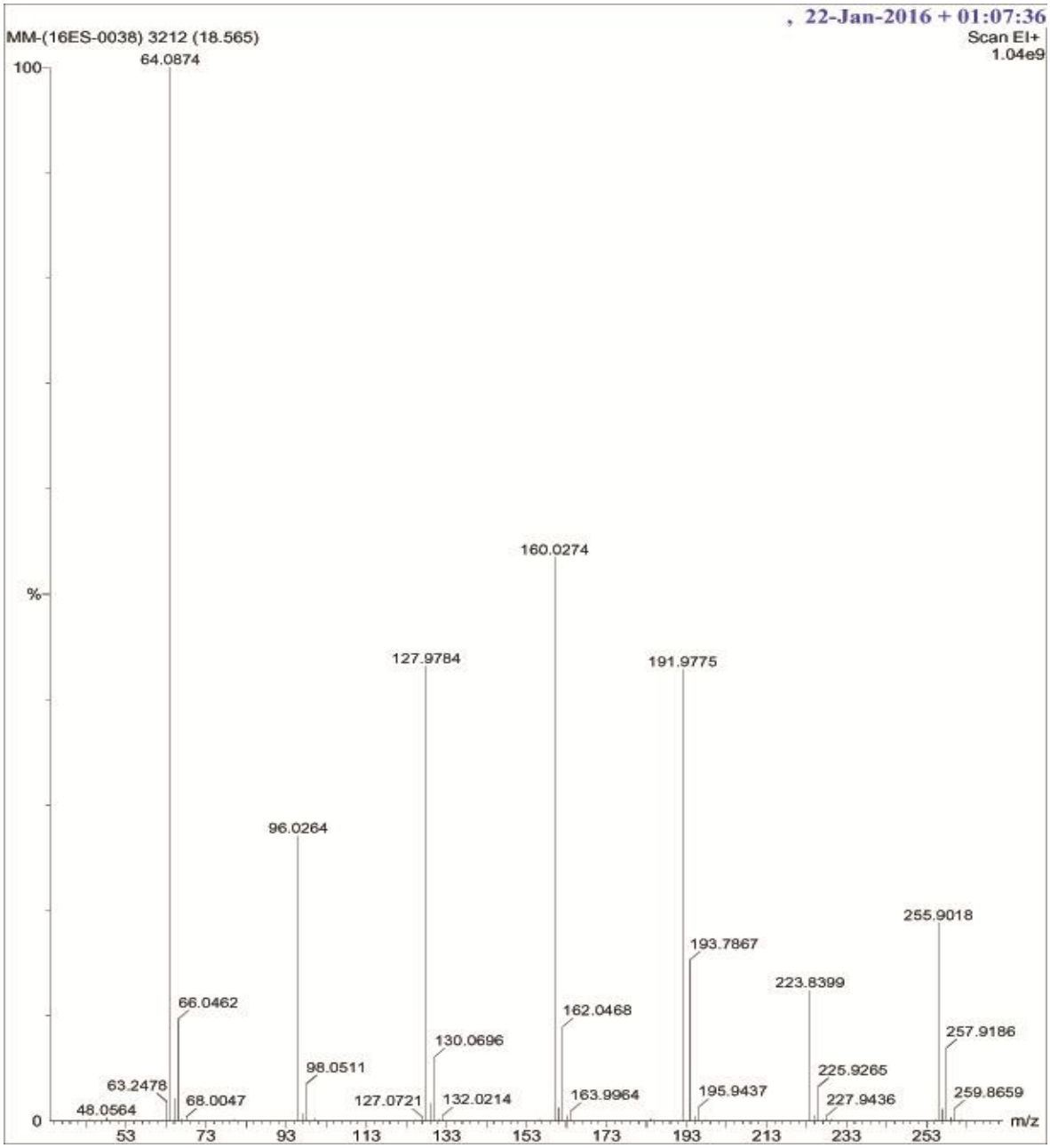
, 22-Jan-2016 + 01:07:36
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100
%
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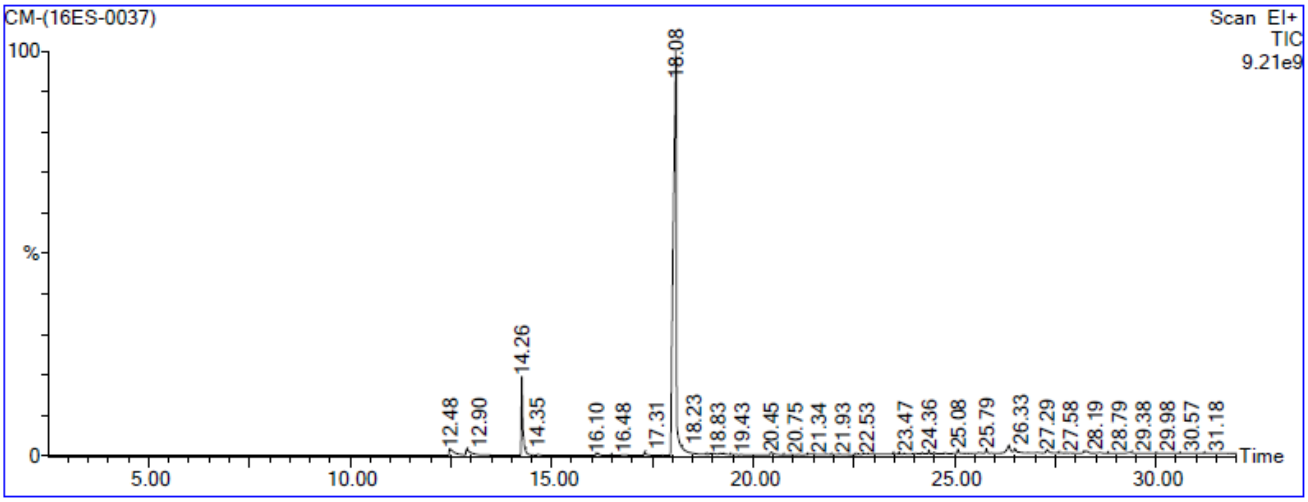
64.0874
160.0274
127.9784
191.9775
96.0264
255.9018
193.7867
223.8399
66.0462
162.0468
257.9186
130.0696
98.0511
225.9265
63.2478
68.0047
127.0721
132.0214
163.9964
195.9437
227.9436
259.8659
48.0564
53
73
93
113
133
153
173
193
213
233
253
m/z

SAMPLE CODE:CM

GC/MS Method: GC: METHOD-1.mth MS: METHOD-1.EXP
Sample ID: CM-(16ES-0037)

Page 1 of 1
Vial Number: 74



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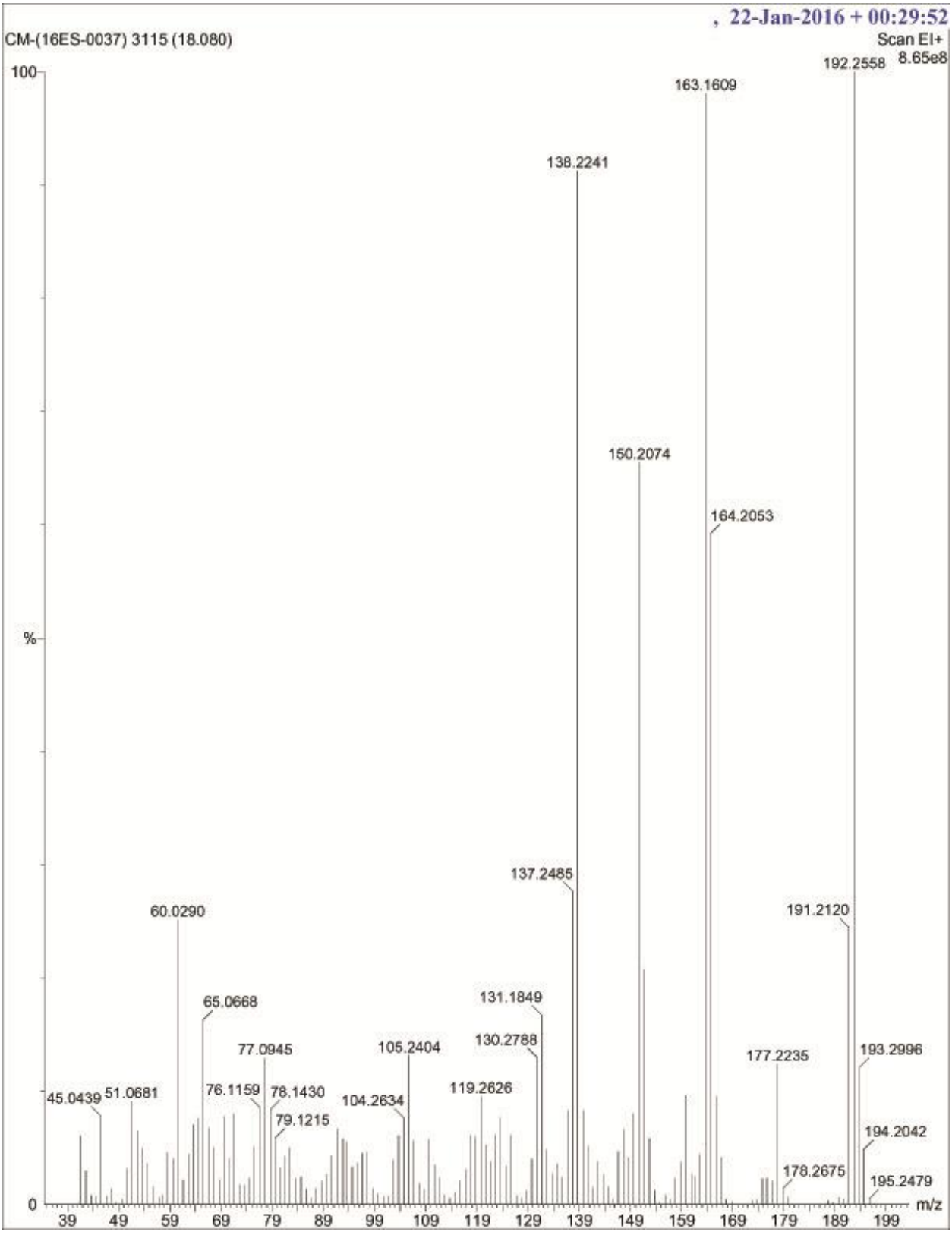
1	14.263	2352	1,770,090,752	70,931,392.0	7.973	8.66
2	18.080	3115	9,152,473,088	818,765,312.0	92.027	100.00


, 22-Jan-2016 + 00:29:52
CM-(16ES-0037) 3115 (18.080)
Scan EI+
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100
%
0
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163.1609
138.2241
150.2074
164.2053
137.2485
60.0290
191.2120
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131.1849
130.2788
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178.2675
195.2479
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159
169
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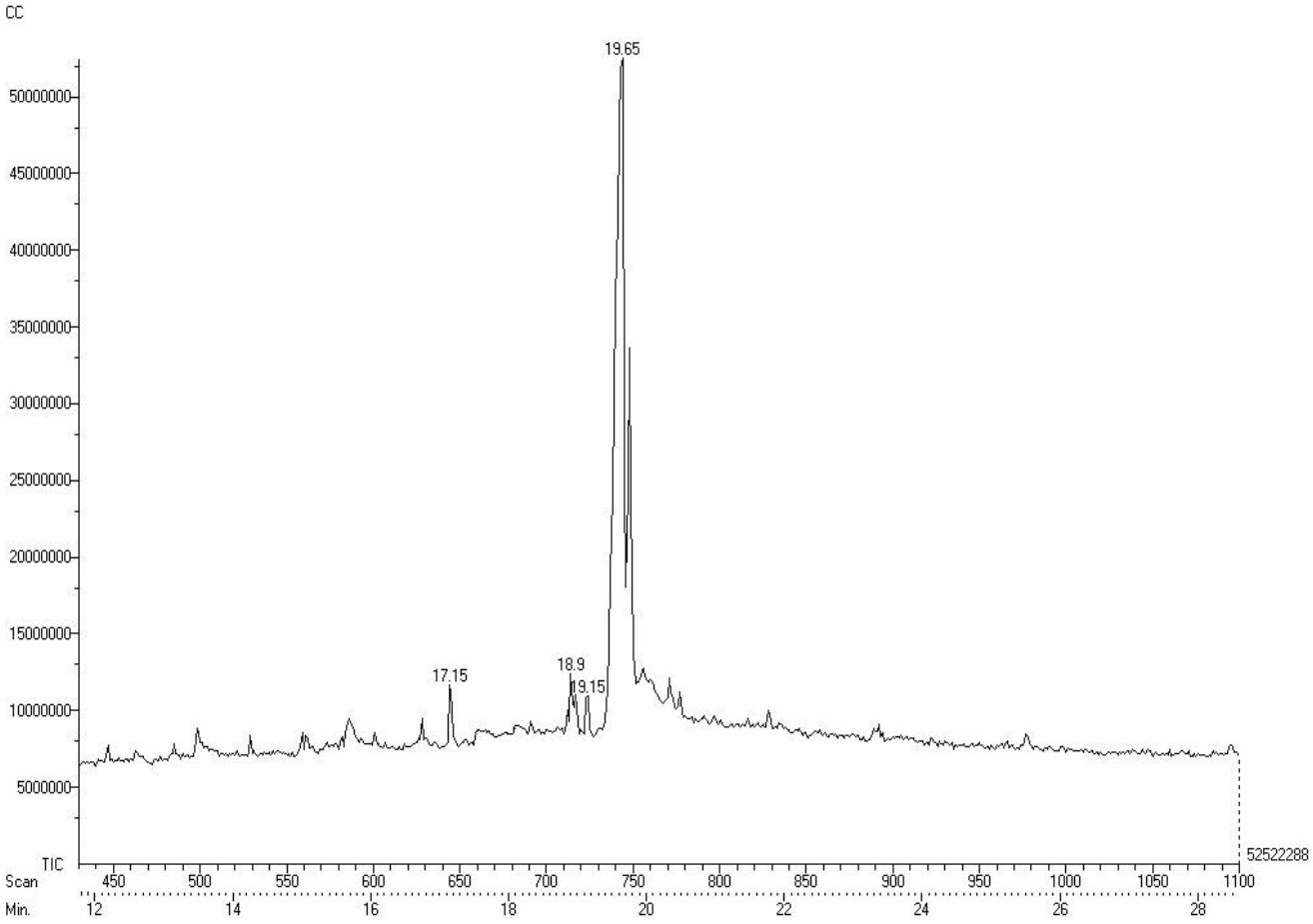
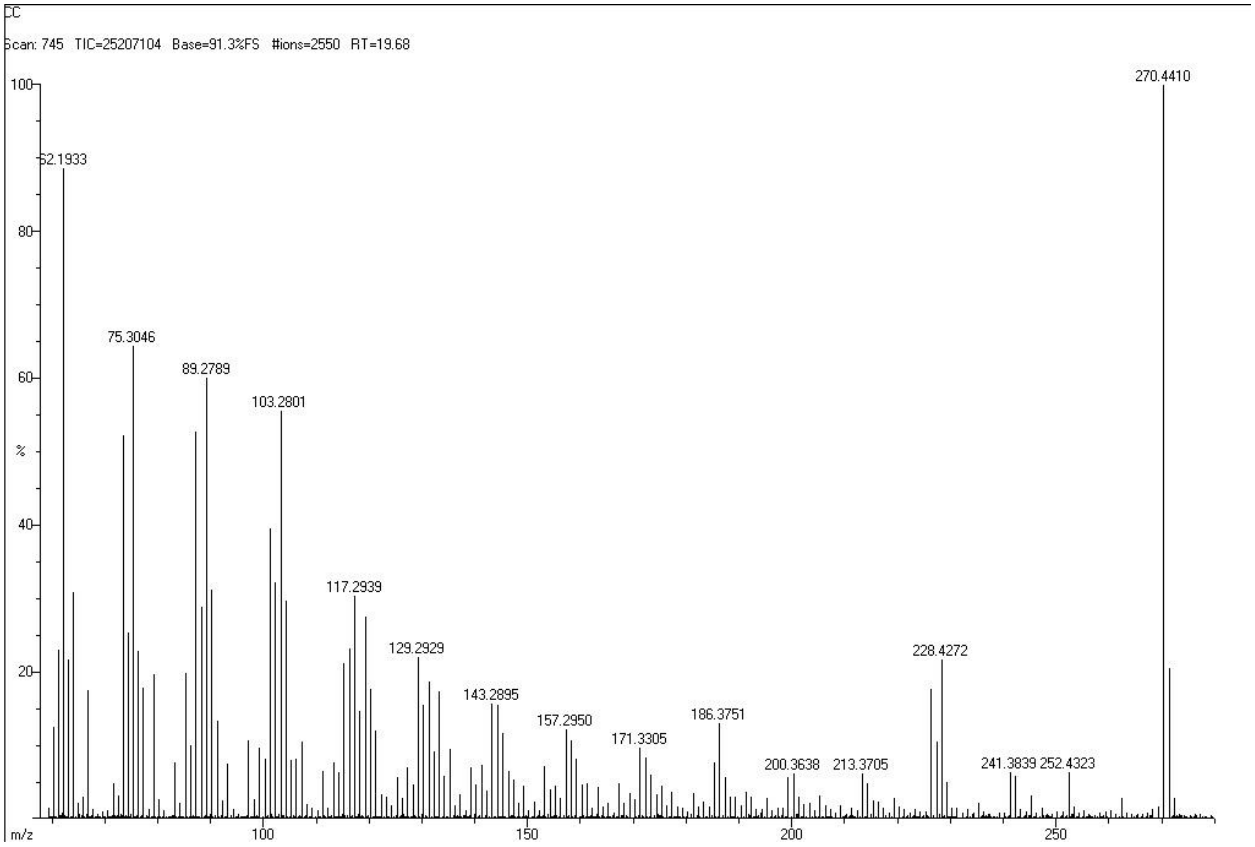
189
199
m/z

SAMPLE CODE: CC




CC
Scan: 745 TIC=25207104 Base=91.3%FS #ions=2550 RT=19.68
100
80
60
40
20
%
m/z
100
150
200
250
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52.1933
75.3046
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103.2801
117.2939
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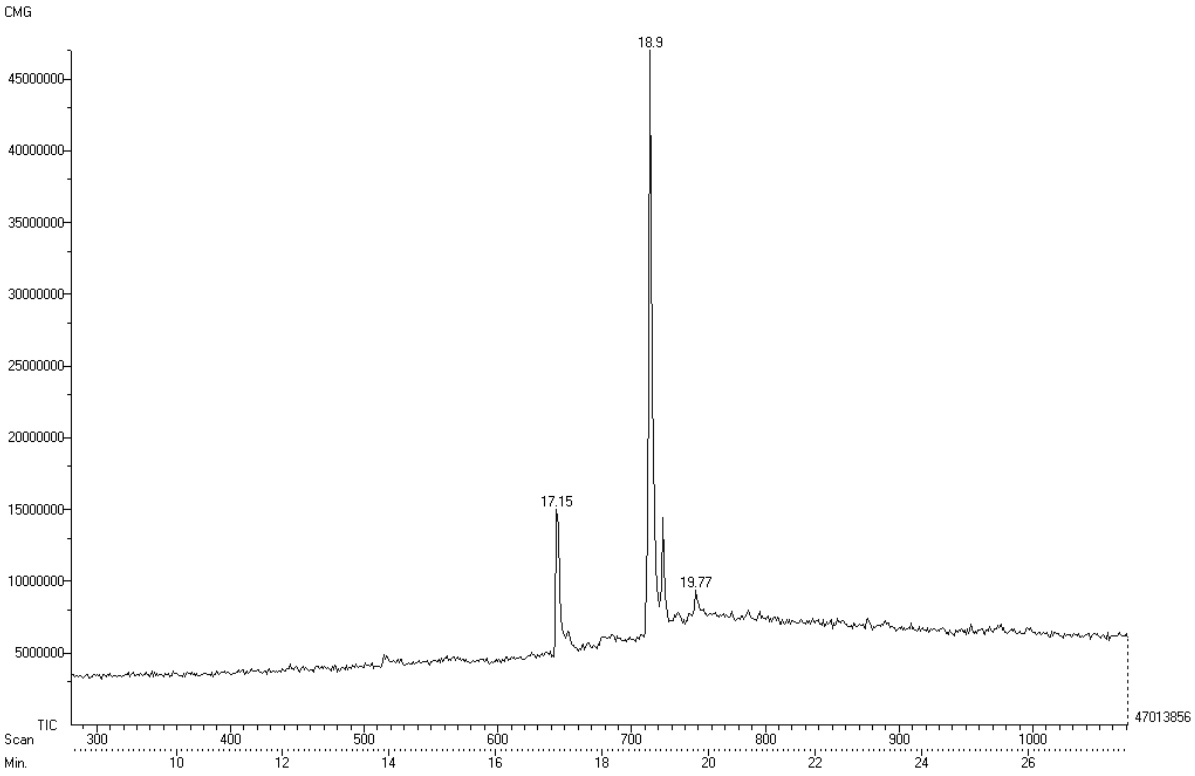
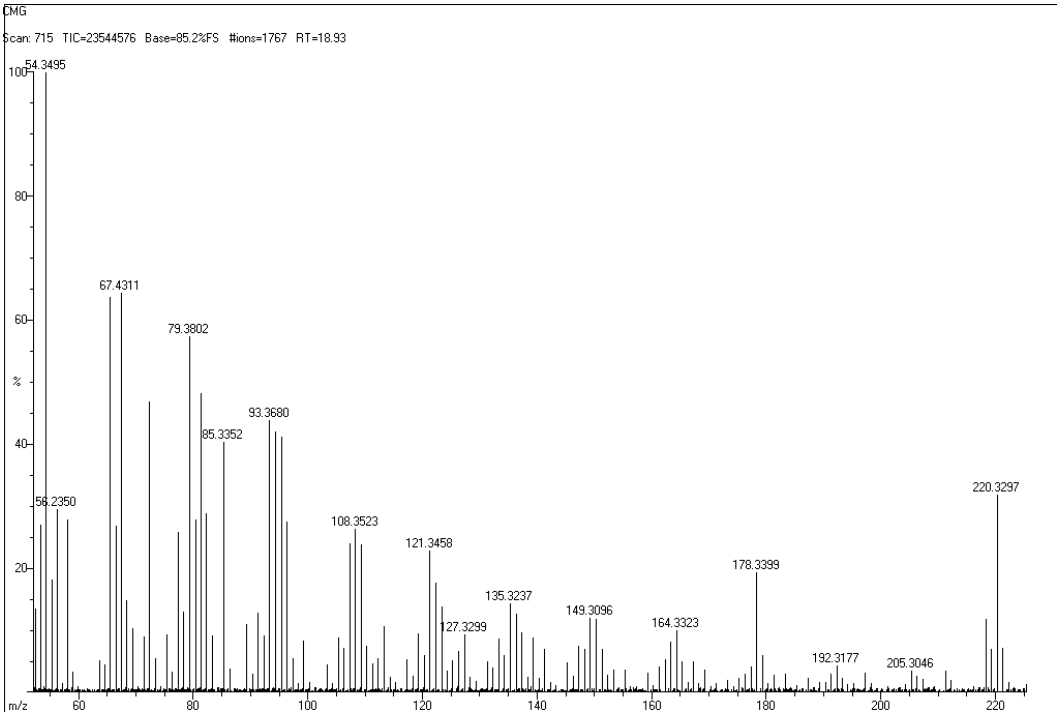
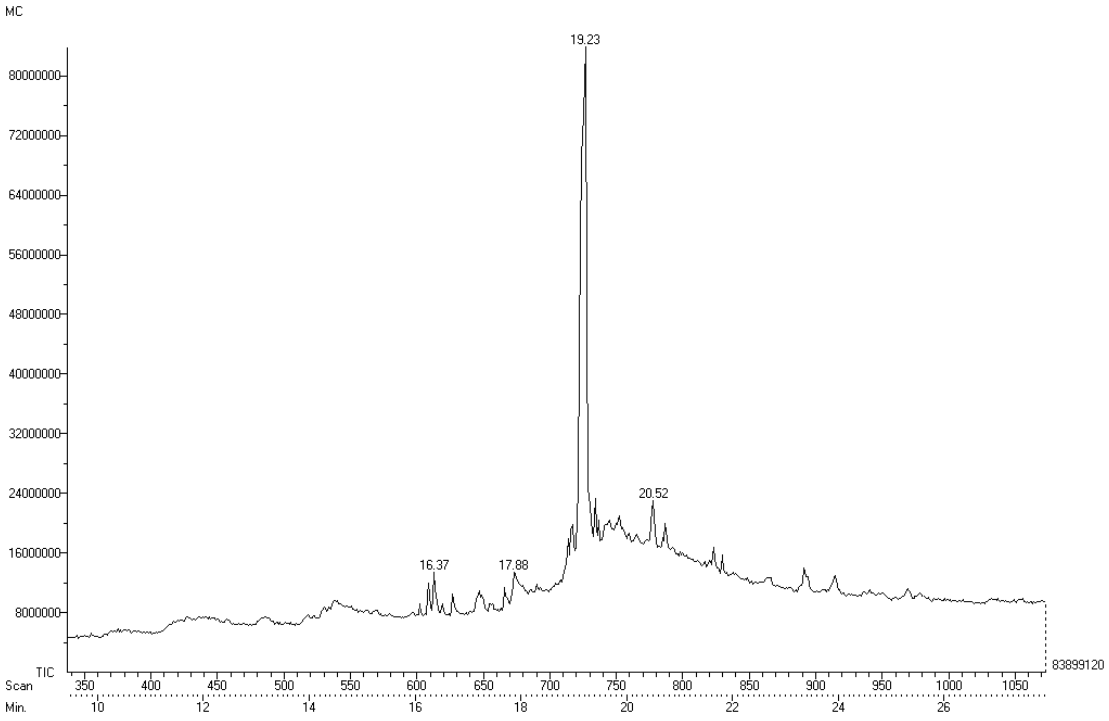
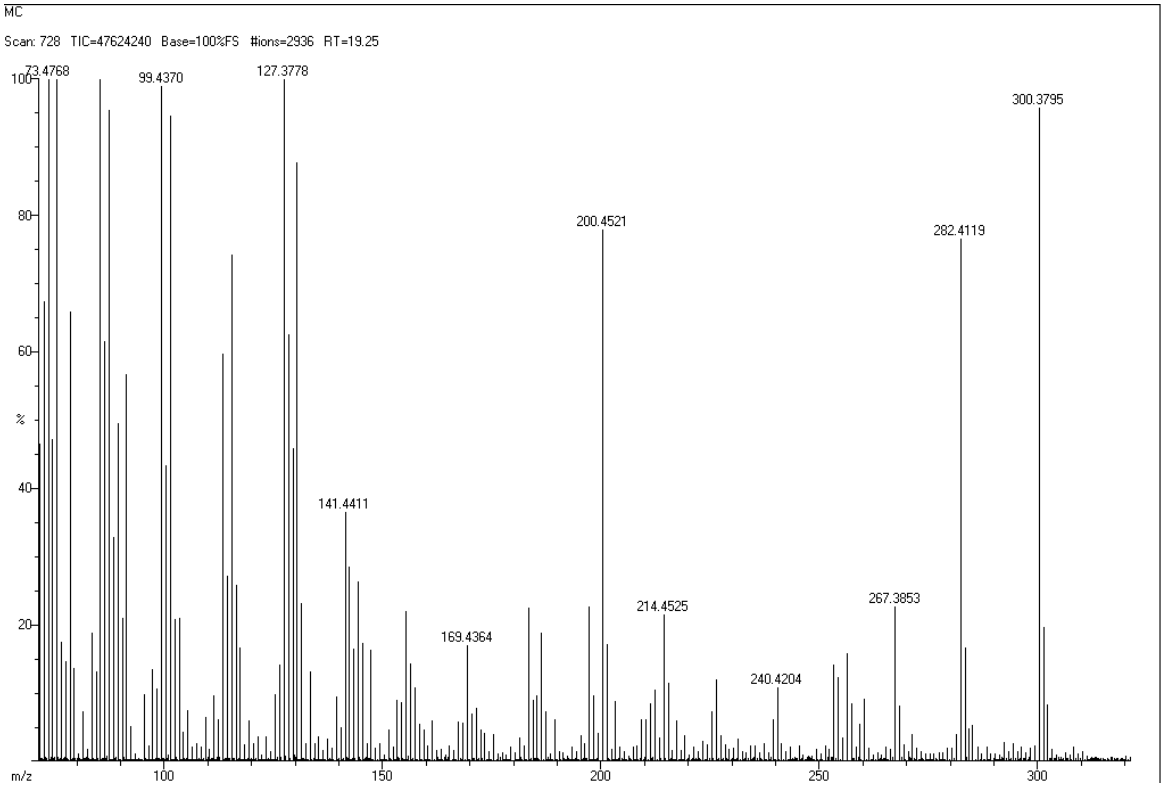
SAMPLE CODE: CMG



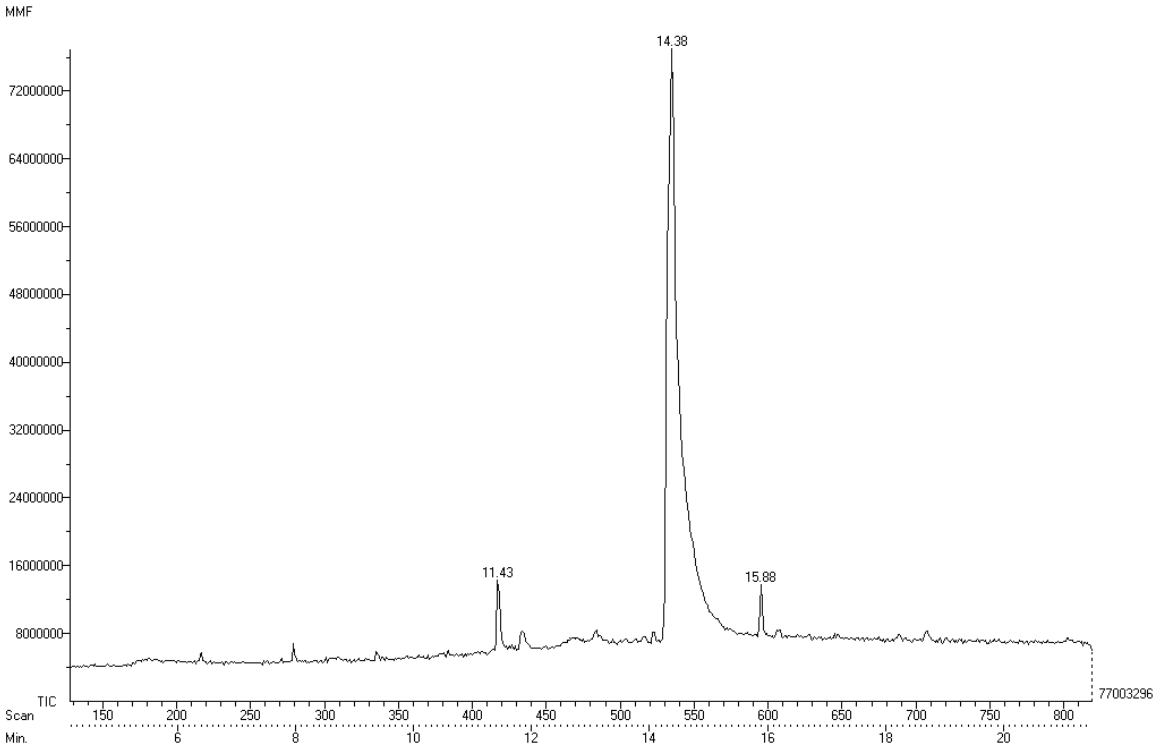


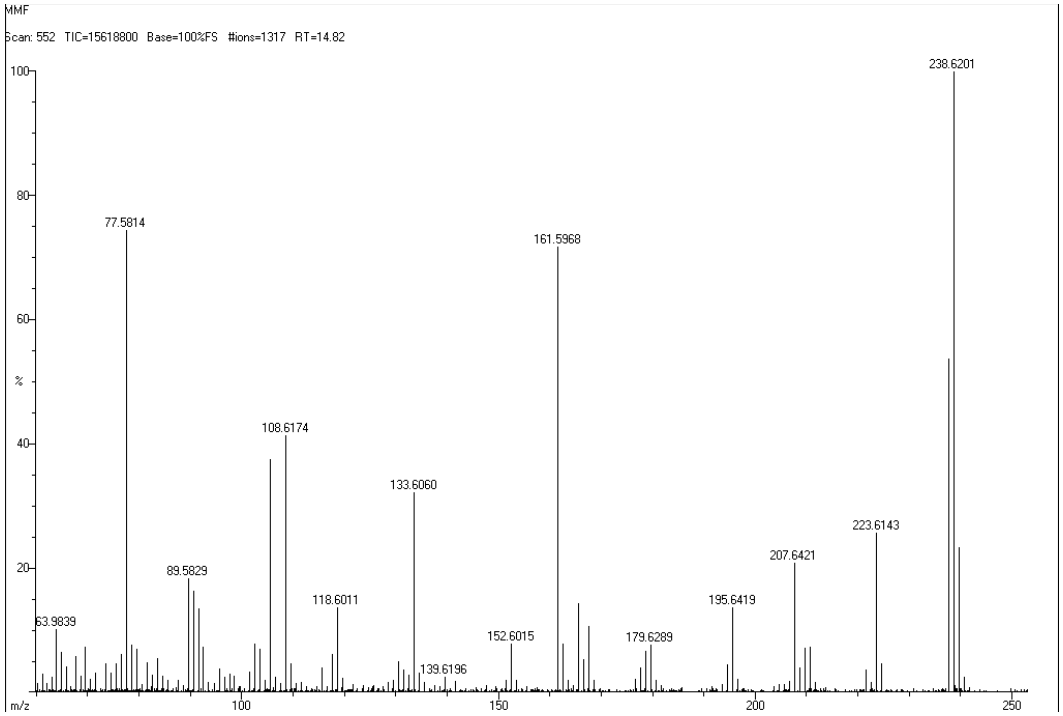
SAMPLE CODE: MC



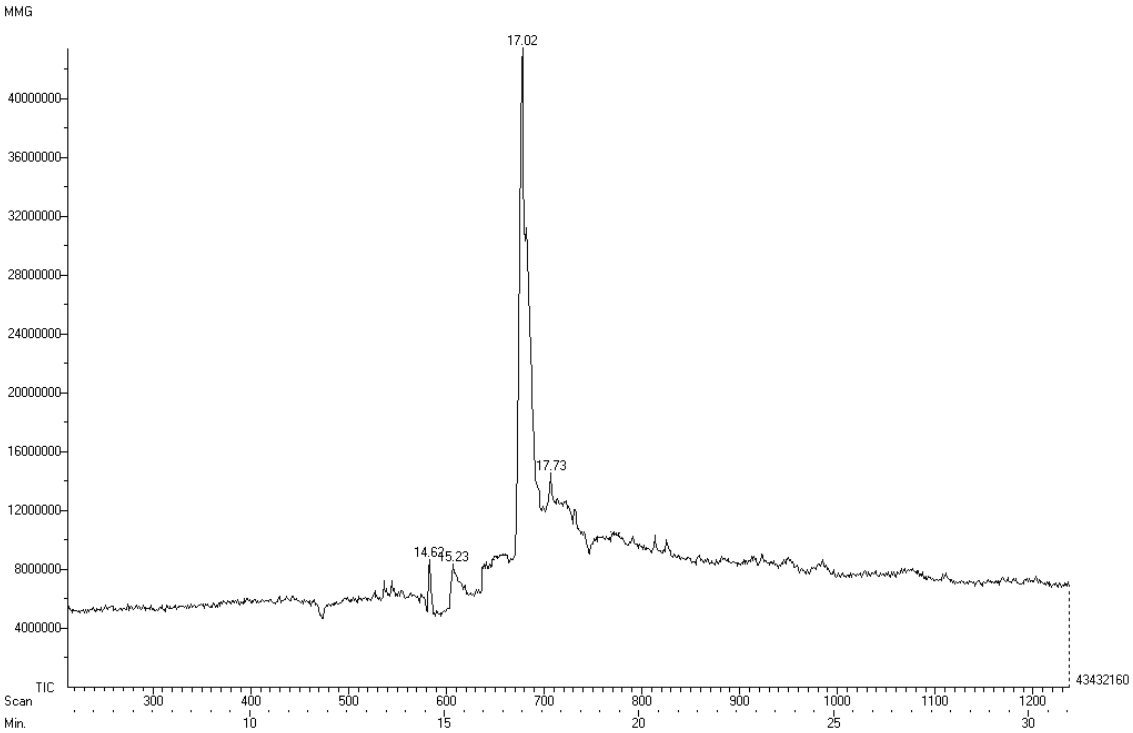


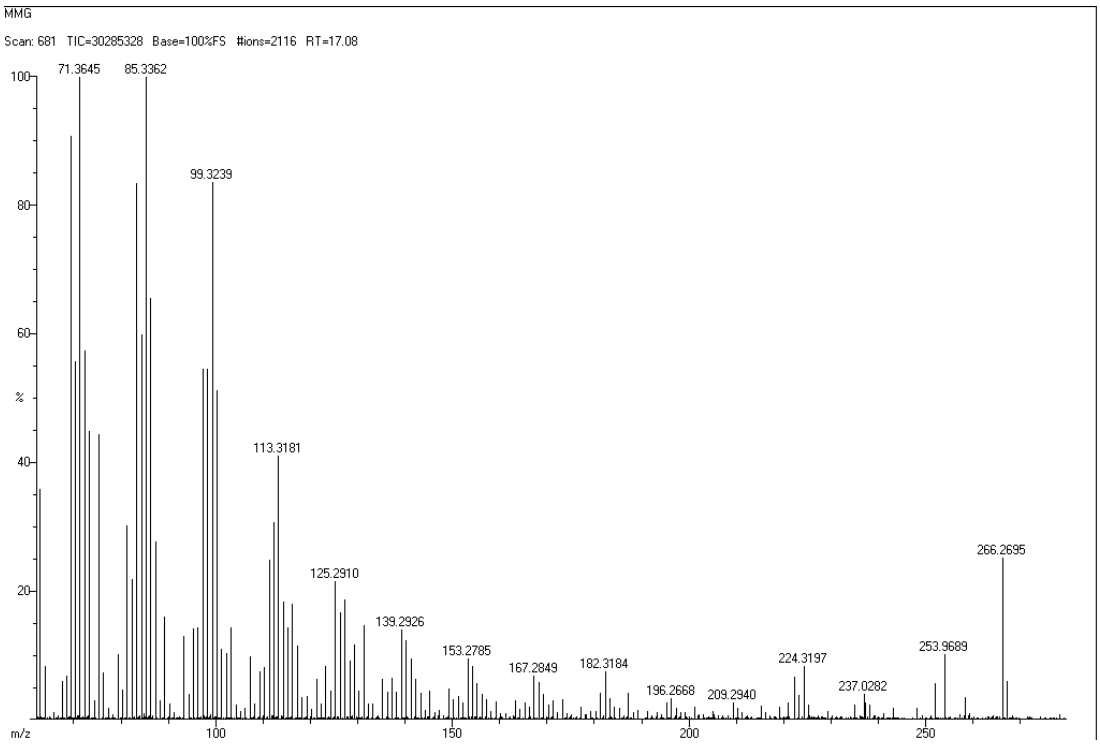
SAMPLE CODE: MMF





SAMPLE CODE: MMG



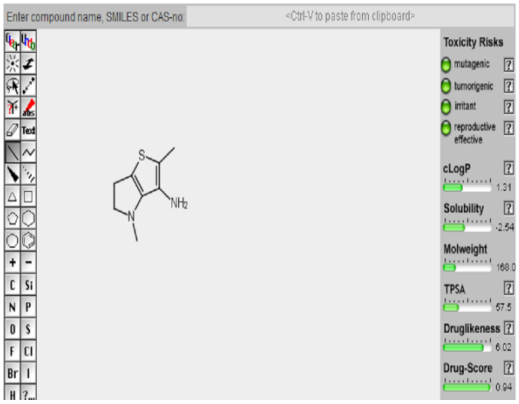


IN-SILICO TOXICITY PREDICTION

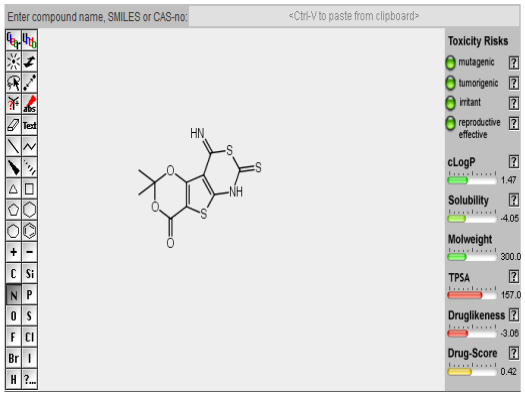
Insilico toxicity prediction was done for the filtered 7 compounds using OSIRIS Property Explorer. This software is available for access in the Organic Chemistry Portal. Using the prediction tool, mutagenicity, tumorogenicity, skin irritation and reproductive effects were calculated. The results were color coded. The green color represents that the compounds is non-toxic. Yellow color indicates moderate and severe toxicity of the chemicals respectively.

TABLE NO 5: TOXICITY PREDICTION

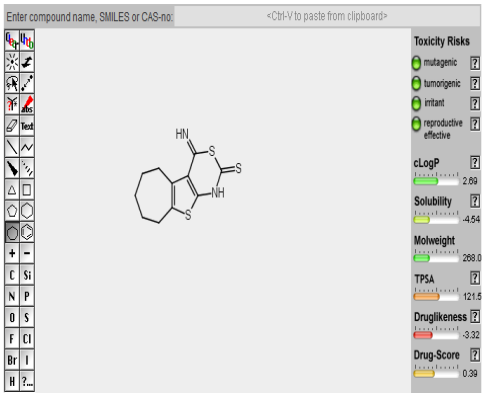
SAMPLE	MM	CM	MMG	MMF	CMG	CC	MC	TM
Mutagenic	+	+	+	+	+	+	+	+
Tumorigenic	+	+	+	+	+	+	+	+
Irritant	+	+	+	+	+	+	+	+
Reproductive effect	+	+	+	+	+	+	+	+



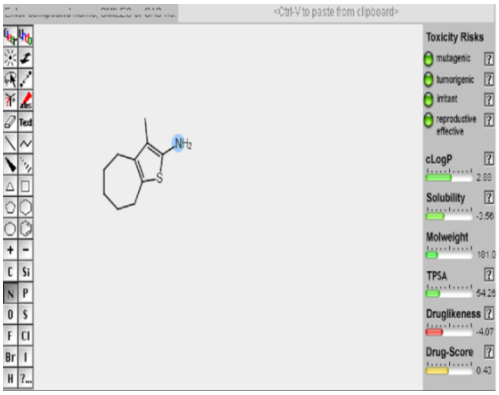
TOXICITY PREDICTION OF TM



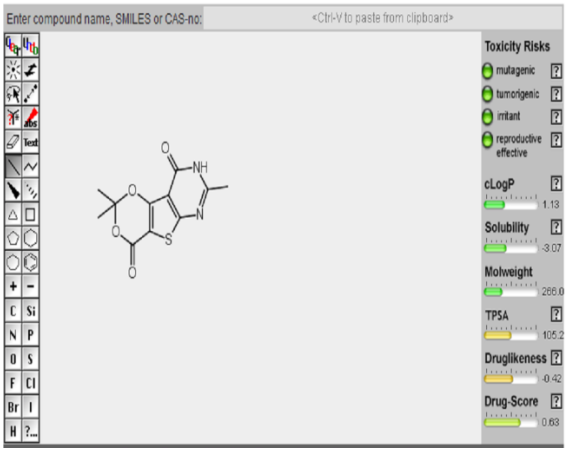
TOXICITY PREDICTION OF MC



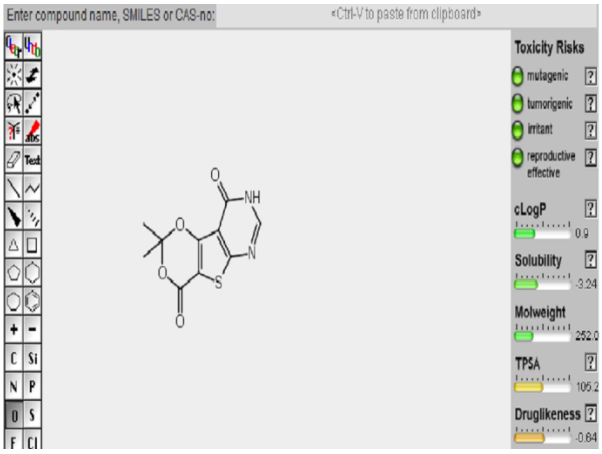
TOXICITY PREDICTION OF CC



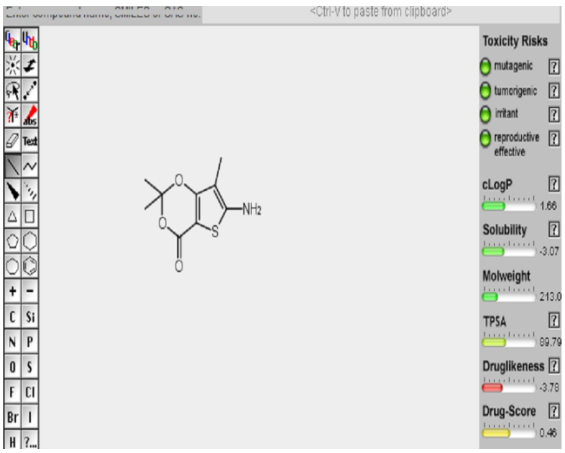
TOXICITY PREDICTION OF CM



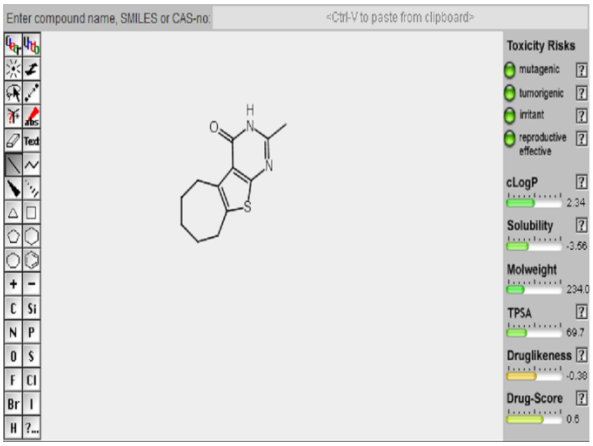
TOXICITY PREDICTION OF MMG



TOXICITY PREDICTION OF MMF



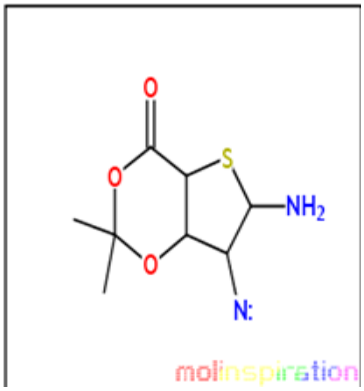
TOXICITY PREDICTION OF MM



TOXICITY PREDICTION OF CMG

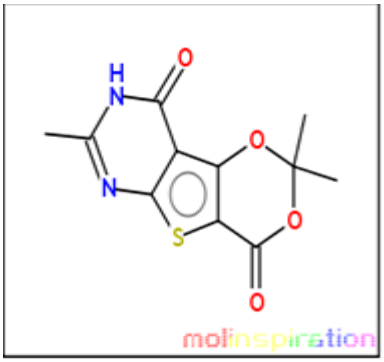
INSILICO SCREENING OF DRUG LIKENESS

COMPOUND NAME: MM



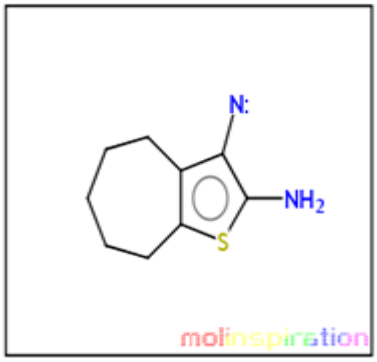
Molinspiration bioactivity score	v2014.03
GPCR ligand	-0.99
Ion channel modulator	-0.60
Kinase inhibitor	-1.31
Nuclear receptor ligand	-1.04
Protease inhibitor	-0.62
Enzyme inhibitor	-0.13

COMPOUND NAME: MMG



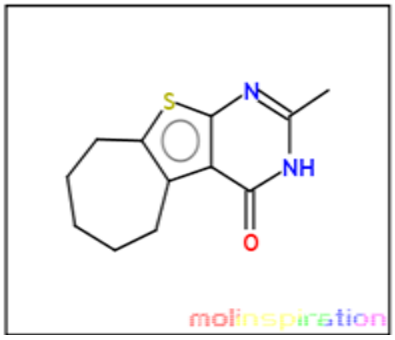
miLogP	1.55
TPSA	81.29
natoms	18
MW	266.28
nON	6
nOHNH	1
nviolations	0
nrotb	0
volume	210.86

COMPOUND NAME: CM



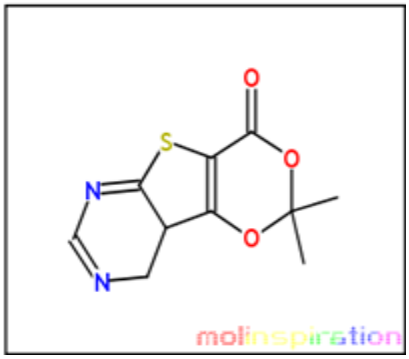
miLogP	2.75
TPSA	48.29
natoms	12
MW	180.28
nON	2
nOHNH	2
nviolations	0
nrotb	0
volume	165.00

COMPOUND NAME : CMG



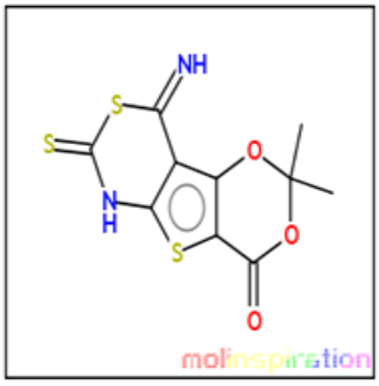
miLogP	2.51
TPSA	45.75
natoms	16
MW	234.32
nON	3
nOHNH	1
nviolations	0
nrotb	0
volume	208.29

COMPOUND NAME: MMF



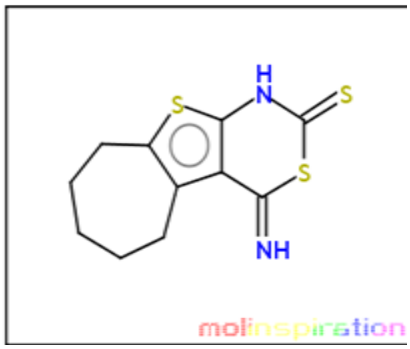
miLogP	1.76
TPSA	60.27
natoms	16
MW	238.27
nON	5
nOHNH	0
nviolations	0
nrotb	0
volume	192.38

COMPOUND NAME: MC



miLogP	1.09
TPSA	75.18
natoms	18
MW	300.39
nON	5
nOHNH	2
nviolations	0
nrotb	0
volume	220.34

COMPOUND NAME: CC



miLogP	2.06
TPSA	39.64
natoms	16
MW	268.43
nON	2
nOHNH	2
nviolations	0
nrotb	0
volume	217.77

BIOLOGICAL EVALUATION

The anti-tubercular activities of the synthesized compounds were determined by Microplate Alamar Blue Assay method (MABA). The organism used in the study is Mycobacterium tuberculosis H37Rv

All the synthesized compounds showed anti-mycobacterial activity in a varying degree against the organism tested. The organism tested was susceptible to all the synthesized compounds and the minimum inhibitory concentration for the compounds varied between 6.25 and 1.6 mcg/ml. The data pertaining to these observations are presented in the table. Inhibition was compared using Pyrazinamide- 3.125 mcg/ml and streptomycin- 6.25 mcg/ml as standard.

Among the synthesized compounds, compound- MMF exhibit activity at 1.6 mcg/ml and compound –TM exhibit activity at 3.12 mcg/ml.

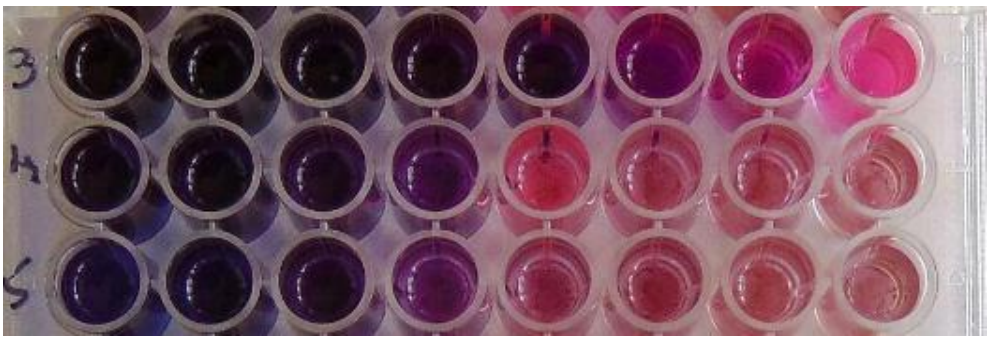
TABLE NO 6: BIOLOGICAL EVALUATION OF SYNTHESIZED COMPOUNDS

Samples	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
CMG	S	S	S	R	R	R	R	R
MMF	S	S	S	S	S	S	S	R
MMC	S	S	S	S	S	R	R	R
TM	S	S	S	S	S	S	R	R

S – SENSITIVE

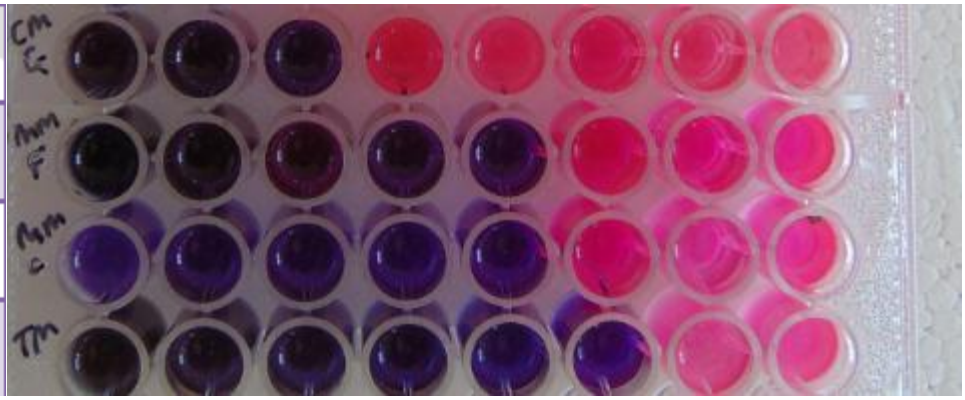
R – RESISTANT

S.NO	SAMPLES	100µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8
1.	CM	S	S	S	S	S	R	R	R
2.	MMG	S	S	S	S	R	R	R	R
3.	MM	S	S	S	S	R	R	R	R



SAMPLE 3: CM, SAMPLE 4: MMG, SAMPLE 5: MM

Sl. No.	Samples	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
	CMG								
	MMF								
	MMC								
	TM								



DISCUSSION

The discussion of the following results given below;

The following compounds have best docking score against specific targets and it was synthesized at appropriate manner , then the purity of the compounds are determined, it shows sharp melting point, single spot obtained in the TLC.

Among 8 compounds, 5 of them were obtained at 95% purity. It was confirmed by GC-MS analysis (obtaining a single peak) and molecular weight also obtained at ± 1 variation.

Then the functional group determination is obtained from FT-IR. It is confirmed by obtaining specific absorption band in the spectra.

Molecular weights of the synthesized compounds are interpretated by NMR, depending upon the no of protons.

The biological evaluation of the compounds are denoted that the specific organism was sensitive at 3.12 and 3.12mcg/ml and showed better activity compared to standard drugs.

The toxicity of the compounds also showed that all the 8 compounds are non-toxic.

SUMMARY

Glutamine synthetase 1 is a vital enzyme present in the cell wall of Mycobacterium tuberculosis H37Rv. It belongs to the Ligase family.

This enzyme was chosen as the target for the drug design study after thorough literature review. A database of 200 molecules with potential to inhibit the target (PDB id: 4 ACF) was chosen by altering the lead molecules, amino thiophene.

The designed molecules were docked against the target chosen using ARGUS LAB.

From among the docked molecules, 8 molecules with good Score were chosen for laboratory synthesis. The drug likeness and toxicity prediction was carried out for the filtered 8 compounds in silico.

Then further the compounds were synthesized. The reaction conditions were optimized.

The compounds were labeled as MM, CM, MMF, CMG, MMG, MC, CC,TM. These compounds were synthesized and recrystallized.

PURITY:

The purity of the compounds was evaluated by sharp melting point and TLC and was characterized by Infrared Spectroscopy, Nuclear Magnetic Resonance Spectroscopy and MASS Spectroscopy. A single peak obtained from GC-MS analysis predicted that the synthesized compounds are formed at correct manner. The molecular weight of the compounds are also determined.

Further, compounds are characterized by IR, NMR (H1NMR) functional groups , number of protons are determined respectively.

All the synthesized compounds exhibited molecular ion peak (M^+) of varying intensities ascertaining the molecular weights of the compounds.

ANTI-MICROBIAL ACTIVITY

The purified compounds were screened for anti-tubercular activity by *invitro* Micro Plate Alamar Blue Assay. It showed that the synthesized compounds are sensitive at 3.12- 1.6 mcg/ml level. So the following results concluded that the synthesized compounds have better anti-tubercular activity.

The synthesized compounds were active at 3.12-1.6 mcg/ml, which are compared to that of the known anti-tubercular agents, Pyrazinamide: 3.125mcg/ml, Ciprofloxacin: 3.125 and Streptomycin: 6.25mcg/ml

TOXICITY PREDICTION

The toxicity of the synthesized compounds are screened by OSIRIS, while it is non-toxic means it showed green color and toxic means it indicated by red. All the compounds are non-toxic and it showed better activity.

CONCLUSION

It is concluded that the synthesized compounds might effectively inhibit the chosen target Glutamine Synthetase I which is essential for the Mycobacterial Tuberculosis. Further structural modifications of the synthesized compounds will aid in the development of potential molecule against the pathogen.

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